Protein disulphide-isomerase from human placenta and rat liver

Purification and immunological characterization with monoclonal antibodies

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The purification of human placenta and rat liver protein disulphide-isomerase (PDI, EC 5.3.4.1) and the production of a panel of monoclonal antibodies against these proteins are described. The physical and enzymic properties of human PDI and rat PDI were similar; immunological characterization revealed the presence of unique, as well as shared, antigenic determinants. Although purified rat liver PDI was present as three forms differing slightly in M_r value, evidence was presented that the multiple forms represent proteolytic degradation products of a single 59000- M_r species. Purified human PDI had an apparent M_r of 61 200. Two of the monoclonal antibodies against human PDI partially inactivated the enzyme, and one of these in indirect immunoprecipitation led to the precipitation of all glutathione:insulin transhydrogenase activity from a crude extract of human placenta. Results of immunofluorescence experiments with HT-29 human colon carcinoma cells were consistent with localization of PDI in the nuclear membrane and cell cytoplasm.

INTRODUCTION

The formation of disulphide bonds is a major force stabilizing the three-dimensional structure of many extracellular proteins, such as serum albumin, immunoglobulins and procollagen. The studies by Anfinsen and co-workers (DeLorenzo *et al.*, 1966; Fuchs *et al.*, 1967) suggested that the catalyst of disulphide-bond formation and isomerization is a microsomal enzyme that they termed 'disulphide interchange enzyme'. This enzyme, which catalyses disulphide interchange between thiols and protein disulphides, has since been given the systematic name protein disulphide-isomerase (PDI, EC 5.3.4.1).

The reverse reaction, reduction of disulphide bonds in proteins, was shown to be catalysed by thiol:protein-disulphide oxidoreductase (EC 1.8.4.2), also known as glutathione:insulin transhydrogenase (Katzen & Stetten, 1962; Kakiuchi & Tomizawa, 1964). The reductive cleavage of the disulphide bonds of insulin has been implicated as the first step in the degradation of this hormone (Varandani, 1977). Since the discovery of PDI and thiol:protein-disulphide oxidoreductase, there has been considerable controversy about whether these activities are catalysed by the same enzyme protein or by different enzymes (Ansorge *et al.*, 1973; Hillson & Freedman, 1980). However, immunochemical studies with polyclonal antisera against purified bovine PDI and thiol:protein-disulphide oxidoreductase indicated that the two enzymes are immunologically indistinguishable (Bjelland *et al.*, 1983). This evidence, taken together with data on the similarities in their physical and enzymic properties (Lambert & Freedman, 1983), led to the conclusion that PDI and thiol:protein-disulphide oxidoreductase are indeed the same enzyme. The net balance of disulphide-bond formation versus reduction *in vitro* appears to depend upon the oxidation–reduction state of the protein and thiol reactants (reviewed by Freedman,

1984). On the basis of their evidence that PDI activity is concentrated in a microsomal fraction from rat liver, but is detectable only after the membrane integrity is damaged, Lambert & Freedman (1985) have proposed a model in which PDI is loosely associated with the luminal surface of the endoplasmic reticulum. The relative contributions of the two activities of PDI *in vivo* are unknown, although there is evidence that disulphide bonds are rapidly and accurately formed in the endoplasmic reticulum on nascent polypeptide chains of albumin (Peters & Davidson, 1982) and immunoglobulins (Bergman & Kuehl, 1979). Direct evidence that this process is catalysed by PDI has not yet been obtained.

Most of the data on the physical and enzymic properties of PDI have been elucidated by purifying and characterizing the bovine and rat enzymes (reviewed by Morin *et al.*, 1983a). Although PDI has been purified from human liver, and polyclonal antibodies against the human enzyme have been prepared (Tomizawa & Varandani, 1965; Varandani & Nafz, 1970), until recently little attention has been directed towards studying the distribution and properties of PDI in human cells. PDI activity has been demonstrated in cultured human fibroblasts (Morin *et al.*, 1983b; Myllylä *et al.*, 1983), where the level of activity was correlated with rates of procollagen synthesis. The availability of many human cell lines and primary cultures producing a variety of disulphide-linked proteins should provide opportunities for characterizing the properties and physiological activities of human PDI.

In the present paper we describe the purification and characterization of PDI from human placenta, as well as preparation of a panel of monoclonal antibodies specific for human PDI. Parallel experiments were carried out with rat liver PDI to facilitate a comparison of the two enzymes. Significantly, two of the anti-(human PDI) monoclonal antibodies were shown to inhibit PDI activity *in vitro*, and may prove to be useful tools for

Abbreviation used: PDI, protein disulphide-isomerase.

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modifying PDI activity *in vivo* in cultured human cells. Monoclonal antibodies against human PDI were used to study the distribution of the enzyme in human colon carcinoma cells by immunofluorescence.

EXPERIMENTAL

Materials

Biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) unless otherwise noted. Na¹²⁵I was from Amersham Corp. (Arlington Heights, IL, U.S.A.) and lactoperoxidase for protein iodination was from Calbiochem-Behring (La Jolla, CA, U.S.A.). Colour-development reagents for immunoblotting were obtained from Kirkegaard and Perry Laboratories (Gaithersburg, MD, U.S.A.). All chemicals for electrophoresis and horseradish-peroxidase-conjugated goat anti-(rabbit IgG) antibody were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Nitrocellulose for immunoblotting was from Millipore Corp. (Bedford, MA, U.S.A.). Alkaline-phosphatase-conjugated goat anti-(mouse IgG) antibody and fluorescein-conjugated rabbit anti-(mouse IgG) antibody were from Zymed Laboratories (South San Francisco, CA, U.S.A.) and Cooper Biomedical (Malvern, PA, U.S.A.) respectively. Sources of other immunological reagents are noted below under 'Methods'. Protein A-containing *Staphylococcus aureus* cells (Pansorbin) were obtained from Calbiochem-Behring. All reagents for cell culture were obtained from GIBCO (Grand Island, NY, U.S.A.). BALB/c mice for hybridoma production were from Cumberland View Farms (Clinton, TN, U.S.A.). Sprague-Dawley rats were obtained from Zivic Miller (Allison Park, PA, U.S.A.).

Methods

Purification of PDI. PDI was purified from human placenta and rat liver by the method of Carmichael *et al.* (1977) with the following modifications. Fresh human placenta (1200 g) was dissected to remove connective tissue, cut into 2.5 cm (1 in) cubes, washed with ice-cold 0.1 M-sodium phosphate buffer, pH 7.5, containing 5 mM-EDTA, diluted with 2 vol. (w/v) of the same buffer containing 1% (v/v) Triton X-100, and homogenized for 30 s with a Brinkmann Polytron tissue disrupter. Homogenates were then sonicated for 30 s at 24 cycles/s. Rat liver (500 g) was homogenized as described by Carmichael *et al.* (1977), except that a 1:2 (w/v) ratio of tissue to buffer was used. The remaining steps in the purification procedure followed the protocol of Carmichael *et al.* (1977), modified by omitting the gel-filtration step in the purification of human placenta PDI. After chromatography on DEAE-Sephadex A-50, the preparations of human PDI and rat PDI were homogeneous by SDS/polyacrylamide-gel electrophoresis, except for minor contamination with serum albumin. Residual albumin was removed by immunoadsorption with anti-(human serum albumin) or anti-(rat serum albumin) antibodies coupled to CNBr-activated Sepharose 4B.

Assays. PDI activity was measured by the glutathione:insulin transhydrogenase assay (Carmichael *et al.*, 1977), modified by omitting the bovine serum albumin from the assay buffer and decreasing the GSH concentration to 0.5 mM. In addition, aprotinin was added to the assay buffer at a concentration of 25

kallikrein-inhibitor units/ml to inhibit proteolytic degradation of ¹²⁵I-insulin. We found that bovine serum albumin, which contains intrachain disulphide bonds, inhibited glutathione:insulin transhydrogenase activity in a dose-dependent fashion, possibly by competing with bovine insulin as a source of disulphide-containing protein substrate (results not shown). Preliminary experiments also indicated that lowering the GSH concentration from 1 mM to 0.5 mM decreased non-specific reduction of insulin (in the absence of enzyme), with no effect on insulin reduction in the presence of enzyme (results not shown). Bovine insulin was labelled with ¹²⁵I by the method of Thorell & Johansson (1971) and stored at -70 °C.

Solid-phase enzyme-linked immunosorbent assays were used to measure serum antibody titres of immunized mice, and to test for specific monoclonal antibody in culture supernatants from cloned hybridomas. Assays were performed as described previously (Kaetzel & Ray, 1984).

Total protein was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Electrophoresis. SDS/polyacrylamide-gel electrophoresis was carried out by the method of Laemmli (1970), with 5% polyacrylamide stacking and 10% polyacrylamide separating slab gels at room temperature. When samples were separated under reducing conditions, dithiothreitol was added to the prepared samples at a final concentration of 0.1 M. Proteins were detected by staining with Coomassie Brilliant Blue R-250 (0.5 g/l in water/methanol/acetic acid, 520:473:57, by vol.) or AgNO₃ (Oakley *et al.*, 1980).

Preparation of monoclonal antibodies. BALB/c mice were immunized with purified human or rat PDI, and hybridomas were constructed by fusion of spleen cells from immunized mice with SP-2/0 myeloma cells, essentially as described by Kaetzel *et al.* (1984). When colonies of cloned hybridomas became visible, culture supernatants were tested for the presence of monoclonal antibodies specific for human or rat PDI by enzyme-linked immunosorbent assay. Positive clones were expanded and subcloned twice by limiting dilution. Monoclonal antibodies secreted by stable subclones isolated in this manner were tested for reactivity against human PDI and rat PDI as well as human serum albumin and rat serum albumin. The isotype of each monoclonal antibody was determined by double immunodiffusion in agar, with isotype-specific anti-(mouse immunoglobulin) antibodies as precipitating reagents (Miles Scientific, Elkhart, IN, U.S.A.). Hybridoma cells (10⁶ cells/mouse) were injected intraperitoneally into BALB/c mice that had previously been primed by intraperitoneal injection of 0.5 ml of 2,6,10,14-tetramethylpentadecane (pristane). Ascites fluids were clarified by centrifugation, and used without further purification for immunoblotting and enzyme-inhibition studies.

Immunoblotting. Purified human PDI and rat PDI (2.5 µg) were electrophoresed in reducing SDS/polyacrylamide gels as described above, then transferred electrophoretically to nitrocellulose membranes essentially as described by Towbin *et al.* (1979). In one experiment, rat liver was homogenized in ice-cold

0.1 M-sodium phosphate buffer, pH 7.5, containing 1% (v/v) Triton X-100, 1 mM-ethylene glycol tetra-acetate, 0.5 mM-phenylmethanesulphonyl fluoride and 25 kallikrein-inhibitor units of aprotinin/ml. The homogenate was centrifuged at 20000 g_{av} for 30 min at 5 °C, and 50 μ g of supernatant protein was processed for SDS/polyacrylamide-gel electrophoresis. The membranes were then incubated with the appropriate monoclonal antibody [ascites fluid diluted 1:500 (v/v) in 1% (w/v) gelatin/phosphate-buffered saline (0.14 M-NaCl/0.01 M-sodium phosphate buffer, pH 7.2)] for 1 h at room temperature. In one experiment, rabbit antiserum against bovine PDI (Morin *et al.*, 1983b; a gift from Dr. J. E. Dixon) was used as primary antibody (diluted 1:100 in 1% gelatin/phosphate-buffered saline). Bound antibody was detected by incubating the membranes with alkaline-phosphatase-conjugated goat anti-(mouse IgG) antibody or horseradish-peroxidase-conjugated goat anti-(rabbit IgG) antibody (1:1000 in 1% gelatin/phosphate-buffered saline). To develop colour, the membranes were incubated in a solution of 5-bromo-4-chloroindol-3-yl phosphate (0.21 g/l) and Nitro Blue Tetrazolium (0.42 g/l) in 0.1 M-Tris/HCl buffer, pH 9.5 (alkaline phosphatase), or 4-chloro-1-naphthol (0.6 g/l) and H_2O_2 (0.015%) in 0.5 M-NaCl/20 mM-Tris/HCl buffer, pH 7.5, containing 20% (v/v) methanol (horseradish peroxidase).

Inhibition of PDI activity *in vitro* by monoclonal antibodies. An extract of human placenta enriched in PDI was prepared according to the purification protocol of Carmichael *et al.* (1977), through chromatography on CM-Sephadex. The preparation was made 20% (v/v) in glycerol and stored at -70 °C; preliminary studies indicated that a preparation stored in this manner lost no PDI activity over a period of several months. The partially purified placenta extract had a total protein content of 12.4 mg/ml and a specific glutathione:insulin transhydrogenase activity of 81 units/mg of protein. Portions (50 μ l) of this placenta extract (50 units of enzyme activity) were mixed with 1, 2.5, 5, 10, 25, 50 or 100 μ l of ascites fluid containing anti-PDI monoclonal antibodies or irrelevant monoclonal antibodies. The volume was adjusted to 250 μ l with 0.1 M-sodium phosphate buffer, pH 7.5, containing 5 mM-EDTA and aprotinin (25 kallikrein-inhibitor units/ml), and the samples were incubated at 37 °C for 30 min. The samples were then transferred to an ice/water bath and mixed vigorously. A 100 μ l portion was removed from each sample and assayed for glutathione:insulin transhydrogenase activity as described above. The soluble immune complexes were then immunoprecipitated by adding 50 μ l of rabbit anti-(mouse IgG) antibody (2 mg of specific antibody/ml; Miles Laboratories, Elkhart, IN, U.S.A.) to each sample and incubating the mixture at 37 °C for 30 min followed by 18 h at 5 °C. Protein A-containing *Staphylococcus aureus* cells were then added (100 μ l of a 1:50 suspension of dried cells per tube), and the samples were incubated for an additional 30 min at 37 °C. The tubes were centrifuged in an Eppendorf Microfuge for 5 min at 5 °C, after which a 100 μ l portion of each supernatant was assayed for glutathione:insulin transhydrogenase activity. Results were expressed as percentage inhibition of enzyme activity relative to a control sample that was incubated in the absence of ascites fluid.

Fast protein liquid chromatography of PDI from an extract of human placenta. To test the possibility that monoclonal antibodies HP13 and HP24 might be recognizing different forms of PDI in human placenta, the extract that was used to study inhibition of PDI activity *in vitro* was chromatographed on a reversed-phase fast-protein-liquid-chromatography column and fractions were tested for immunoreactivity with antibodies HP13 and HP24. A 100 μ l portion of the placenta extract described above for enzyme inhibition experiments (1.24 mg of protein) was diluted to 500 μ l with aq. 0.1% (v/v) trifluoroacetic acid and applied to a 0.5 cm $\times$ 10 cm reversed-phase C_{18} column (ProRPC HR 5/10; Pharmacia Fine Chemicals, Uppsala, Sweden). The column was washed with 4 ml of aq. 0.1% trifluoroacetic acid at room temperature at a flow rate of 0.5 ml/min, then eluted with a gradient of 0–70% (v/v) acetonitrile in aq. 0.1% trifluoroacetic acid. Fractions were freeze-dried and resuspended in 0.5 ml of phosphate-buffered saline/fraction. Portions (50 μ l) of each fraction were adsorbed on the wells of a micro titre plate, then assayed for immunoreactivity with monoclonal

Fig. 1. SDS/polyacrylamide-gel electrophoresis of purified human placenta PDI and rat liver PDI

Purified human placenta PDI and rat liver PDI were subjected to SDS/polyacrylamide-gel electrophoresis under reducing (lanes 1 and 2) or non-reducing (lanes 3 and 4) conditions and stained with Coomassie Blue. Lanes 1 and 3, human placenta PDI; lanes 2 and 4, rat liver PDI. The distances migrated by the M_r markers (not shown) are indicated to the left of the Figure. The M_r markers (reduced) were phosphorylase *b* (M_r 92 500), bovine serum albumin (M_r 68 000), ovalbumin (M_r 43 000) and carbonic anhydrase (M_r 30 000). The beginning of the separating gel is at the top of the photograph.

Table 1. Cross-reactivity of monoclonal antibodies against human placenta and rat liver PDI

Monoclonal-antibody-containing culture supernatants were tested for immunoglobulin isotype by immunoprecipitation in agar with isotype-specific antibodies, and for cross-reactivity against human placenta PDI and rat liver PDI by enzyme-linked immunosorbent assay. The cross-reactivity assay involved adsorption of purified human placenta or rat liver PDI (50 μ l of a 10 μ g/ml solution) to wells of a plastic micro titre plate, followed by incubation with 50 μ l of culture supernatant. Bound antigen-monoclonal-antibody complexes were detected with alkaline-phosphatase-conjugated anti-(mouse IgG) antibody. The absorbance at 410 nm was read after incubation with *p*-nitrophenyl phosphate for 15 min (RL monoclonal antibodies) or 95 min (HP monoclonal antibodies). Monoclonal antibodies with the prefix 'HP' were derived by fusion of spleen cells from mice immunized with human placenta PDI; monoclonal antibodies with the prefix 'RL' were derived by fusion of spleen cells from mice immunized with rat liver PDI.

Monoclonal antibody	Isotype	Immunoreactivity (A_{410})		Time of incubation with substrate (min)
		Against human PDI	Against rat PDI	
HP13	IgG1	0.363	0.369	95
HP24	IgG1	0.373	0.021	95
HP49	IgG1	0.423	0.016	95
HP50	IgG2a	0.696	1.130	95
RL12	IgG2b	0.027	1.368	15
RL16	IgG2a	0.002	1.151	15
RL72	IgG2a	0.349	1.207	15
RL77	IgG2b	0.844	1.418	15
RL90	IgG2a	0.589	1.130	15
RL100	IgG2b	0.019	1.486	15
RL119	IgG2a	0.854	1.412	15

antibodies HP13 and HP24 by enzyme-linked immunosorbent assay (described above). Ascites fluid containing HP13 or HP24 antibody (diluted 1:500 in phosphate-buffered saline containing 1% bovine serum albumin) was used as primary antibody.

Immunofluorescence. Cells of the HT-29 human colon carcinoma cell line (Huang *et al.*, 1976) were grown on glass slides in RPMI-1640 medium containing 10% (v/v) foetal bovine serum. At or near confluence, the cultures were immunostained *in situ* at room temperature, either live in the presence of NaN_3 (to prevent capping) or after fixation for 3 h at room temperature with a mixture containing 10 mM- NaIO_4 , 75 mM-lysine, 2% (w/v) para-formaldehyde and 37.5 mM- Na_2PO_4 , pH 6.2. Fixed cells were permeabilized with 0.05% (w/v) saponin to allow better access of antibodies to intracellular structures. Live or fixed cells were washed with phosphate-buffered saline containing 0.02% NaN_3 , 0.05% saponin (fixed cells only) and 0.1% (w/v) ovalbumin, incubated for 1 h at room temperature with monoclonal-antibody-containing ascites fluid (diluted 1:100 in the same buffer used for washing), washed, incubated for 1 h at room temperature with fluorescein-conjugated goat anti-(mouse IgG) antibody (diluted 1:100), and washed as before. Live cells were post-fixed with acetone for 1 min. The slides were then mounted and viewed with a Leitz Orthoplan microscope equipped for epifluorescence.

RESULTS AND DISCUSSION

PDI was purified from human placenta and from rat liver by the method of Carmichael *et al.* (1977), and their M_r values were estimated by SDS/polyacrylamide-gel electrophoresis (Fig. 1). Under reducing conditions, human placenta PDI migrated as a single band of M_r 61 200, whereas rat liver PDI migrated as a minor band

of M_r 58 900 and two major bands of M_r 55 200 and 53 800. When the purified enzymes were separated by electrophoresis under non-reducing conditions, most of the protein migrated at the M_r of monomeric human PDI and rat PDI, although some disulphide-linked aggregates were observed near the origin.

Purified human placenta PDI and rat liver PDI and rat liver PDI were used to immunize mice, and a panel of hybridomas was generated after fusion of spleen cells from these mice with SP-2/0 cells. The specificities of the monoclonal antibodies were determined by enzyme-linked immunosorbent assay (Table 1) and immunoblotting (Fig. 2). Monoclonal antibodies with the prefix 'HP' were derived from mice immunized with human placenta PDI, and those with the prefix 'RL' were derived from mice immunized with rat liver PDI. The RL monoclonal antibodies bound to a much greater extent to immobilized antigens in enzyme-linked immunosorbent assays than did the HP monoclonal antibodies, for which it was necessary to use a longer incubation with chromogenic substrate (Table 1).

An interesting pattern of cross-reactivity against human placenta PDI and rat liver PDI was observed for both the HP and RL monoclonal antibodies (Table 1 and Fig. 2). Two out of four of the HP monoclonal antibodies cross-reacted with rat liver PDI, and four out of seven of the RL monoclonal antibodies cross-reacted with human placenta PDI. Immunoblotting analysis demonstrated that monoclonal antibodies HP13 and HP50 as well as all the RL monoclonal antibodies bound to each of the three forms of rat liver PDI (Fig. 2 and other results not shown). In addition, rabbit polyclonal antibodies against bovine liver PDI reacted with human placenta PDI and all three forms of rat liver PDI (Fig. 2). Taken together, these results suggest that there are antigenic determinants on the PDI molecule that are conserved among diverse mammalian species, as well as

Fig. 2. Immunoblotting of human placenta PDI and rat liver PDI with polyclonal and monoclonal antibodies

Antigens to be tested for immunoreactivity were separated by electrophoresis in SDS/polyacrylamide gels under reducing conditions, then electrophoretically transferred to nitrocellulose membranes. The membranes were incubated with polyclonal (rabbit) or monoclonal (mouse) anti-PDI antibodies, washed, and incubated with horseradish-peroxidase-conjugated anti-(rabbit IgG) antibody or alkaline-phosphatase-conjugated anti-(mouse IgG) antibody. Colour was developed with the appropriate chromogenic substrate as described in the Experimental section. Antigens: lanes 1, 3, 5, 7, 9 and 12, 2.5 μ g of purified rat liver PDI; lanes 2, 4, 6, 8 and 10, 2.5 μ g of human placenta PDI; lane 11, 50 μ g of a 20000 g supernatant from rat liver homogenized in the presence of 1% Triton X-100 and proteinase inhibitors. Antibodies: lanes 1 and 2, rabbit anti-(bovine liver PDI) (1:100); lanes 3 and 4, HP13 ascites (1:500); lanes 5 and 6, HP24 ascites (1:500); lanes 7 and 8, HP49 ascites (1:500); lanes 9 and 10, HP50 ascites (1:500); lanes 11 and 12, RL16 ascites (1:500). Similar results were observed when human PDI and rat PDI were electrophoresed under non-reducing conditions (results not shown).

species-specific antigenic determinants. In keeping with the concept of structural and functional conservation of PDI, Edman *et al.* (1985) have reported sequence homology between rat PDI and *Escherichia coli* thioredoxin, a protein known to share some of the catalytic properties of PDI (Holmgren *et al.*, 1975; Holmgren, 1979). This homology was most striking when two domains of rat PDI containing the postulated active sites (residues 9–90 and 353–431) were compared with the corresponding domain of thioredoxin (residues 16–84). Within these regions, 51% of the amino acids were identical, and an additional 16% showed functional conservation.

Immunological, biochemical and enzymic analyses indicated that the three forms of rat liver PDI are closely related. One explanation is that the 55200- M_r and 53800- M_r forms are degradation products of the 58900- M_r form generated during purification. To test this possibility, we prepared a 20000 g crude supernatant from rat liver in the presence of Triton X-100 and the proteinase inhibitors EGTA, phenylmethanesulphonyl fluoride and aprotinin. Total proteins from this supernatant were separated by SDS/polyacrylamide-gel electrophoresis, and tested for reactivity against each of the RL monoclonal antibodies by immunoblotting. All of the RL monoclonal antibodies reacted only with a single

band of M_r 59000 (results for antibody RL16 are shown in Fig. 2, lane 11). These results contrast with the findings reported by Dawson *et al.* (1984) and Roth & Mesirrow (1984), who found two forms of PDI in extracts of several rat tissues separated by SDS/polyacrylamide-gel electrophoresis and probed with monoclonal antibodies to rat liver PDI. These investigators, however, did not include proteinase inhibitors in their extraction buffers. Our observations support the conclusion that 'multiple forms' of purified rat liver PDI represent degradation products of a single enzyme species present *in vivo*.

The experiments described above demonstrated that all of our monoclonal antibodies bound to either human or rat PDI, or both, when the antigens were immobilized on a solid support. Because monoclonal antibodies against an enzyme often bind to the enzyme without inhibiting its biological activity (for example see Kaetzel *et al.*, 1984), we were interested in testing the effects of monoclonal antibodies on the enzymic activity of PDI. Ascites fluids containing monoclonal antibody HP13, HP24, HP49 or HP50 were incubated with a crude extract of human placenta that contained PDI, and tested for their ability to inhibit glutathione:insulin transhydrogenase activity in solution (Fig. 3a) or to immunoprecipitate PDI from solution (Fig. 3b). Monoclonal antibodies HP49 and HP50 did not inhibit

Fig. 3. Inhibition and immunoprecipitation of human placenta PDI *in vitro* by monoclonal antibodies HP13 and HP24

Extracts of human placenta containing 50 units of glutathione:insulin transhydrogenase activity were incubated with increasing amounts of ascites fluid containing monoclonal antibodies HP13 (■), HP24 (●), a 1:1 mixture of HP13 and HP24 (▲) or an irrelevant monoclonal antibody (◻). Results are expressed as percentage inhibition of glutathione:insulin transhydrogenase activity relative to a control sample incubated in the absence of ascites fluid. In (a) the results of inhibition of enzyme activity by formation of soluble-PDI-anti-PDI immune complexes are shown. In (b) the soluble immune complexes from (a) were immunoprecipitated with rabbit anti-(mouse IgG) antibody and *Staphylococcus aureus* cells; percentage inhibition of activity was determined by assaying enzyme activity remaining in the supernatants after immunoprecipitation. Complete details of the incubation protocols are given in the Experimental section.

glutathione:insulin transhydrogenase activity, and only weakly immunoprecipitated the enzyme (results not shown). In contrast, both antibody HP13 and antibody HP24 inhibited glutathione:insulin transhydrogenase activity in a dose-dependent relationship, with antibody HP24 being somewhat more effective. An equal mixture of antibodies HP13 and HP24 was more effective in the inhibition of enzyme activity than either antibody alone, suggesting a synergistic interaction. Interestingly, complete inhibition of enzyme activity was not seen with either or both monoclonal antibodies, even at a 10–15-fold molar excess of antibody over antigen (100 µl of ascites fluid). Results from immunoprecipitation experiments (Fig. 3b) suggested that the incomplete enzyme inhibition was not caused by insufficient binding of the monoclonal antibodies to PDI in solution. Monoclonal antibody HP24 or an equal mixture of antibodies HP13 and HP24 led to more than 95% precipitation of PDI at a dose of 10 µl of ascites fluid, representing approximately equimolar concentrations of antigen and antibody. Monoclonal antibody HP13 led to precipitation of 55% of PDI at equivalence, whereas only 10% of enzyme activity was inhibited at this dose.

The synergistic inhibitory effects of monoclonal antibodies HP13 and HP24 on human PDI activity suggested that the two antibodies recognize distinct epitopes on the PDI molecule or, alternatively, that they recognize different forms of the enzyme. If the second possibility were correct, the alternative forms of the enzyme could not differ significantly in either M_r or net charge, since the two antibodies reacted with a protein of identical M_r in immunoblotting experiments and a single peak eluted from DEAE-Sephadex (the final step in the purification of human PDI). To test further the possibility that variant forms of human PDI might exist, we chose a separation technique that had not been utilized in the purification of the enzyme. The crude

extract of human placenta that was used for enzyme inhibition experiments was separated by reversed-phase h.p.l.c., and fractions eluted from this column were tested for immunoreactivity with monoclonal antibodies HP13 and HP24 (Figs. 4a and 4b). The elution profiles of immunoreactive protein were virtually identical for the two antibodies.

When the major peak of immunoreactivity for both antibodies, eluted at 25.5 ml, was subjected to SDS/polyacrylamide-gel electrophoresis, a major band of M_r 62000 was observed, similar to the M_r of purified human PDI (Fig. 4c). The results of this experiment support the conclusion that monoclonal antibodies HP13 and HP24 recognize different epitopes on the same PDI molecule. The epitope recognized by HP13 is apparently more conserved in evolution, since this antibody binds to rat PDI, whereas HP24 does not (Table 1 and Fig. 2).

One explanation for the behaviour of monoclonal antibodies HP13 and HP24 is that they do not react with the active site of PDI, but instead cause a conformational change that partially decreases PDI activity. An alternative explanation is suggested by the studies made by Edman *et al.* (1985) on the sequence of rat PDI, from which they drew the conclusion that PDI may have two active sites, each with a reactive disulphide bond. Our finding that the anti-(human PDI) monoclonal antibodies could cause only 50% inhibition of PDI activity may reflect the ability of these antibodies to bind at or near only one of the two active sites. Further studies with these monoclonal antibodies may yield information on the structure and function of human PDI, and they may also prove to be useful tools for inhibiting PDI activity *in vivo*.

To study the localization of human PDI, we used monoclonal antibody HP24 to stain HT-29 human colon carcinoma cells by immunofluorescence (Fig. 5). Staining of the nuclear membrane was observed, as well as

Fig. 4. Immunoreactivity of monoclonal antibodies HP13 and HP24 against human placental proteins separated by reversed-phase fast protein liquid chromatography

A portion of the extract of human placenta used for inhibition experiments *in vitro* (Fig. 3) (1.24 mg of total protein, 50 units of glutathione:insulin transhydrogenase activity) was applied to a reversed-phase C_1/C_8 fast-protein-liquid-chromatography column and proteins were eluted with a gradient of acetonitrile in aq. 0.1% trifluoroacetic acid. In (a) the absorbance of eluted material at 280 nm is designated by the thick line, and the thin line represents the shape of the acetonitrile gradient (0–75% acetonitrile in a total volume of 30 ml). In (b) eluted fractions were tested for immunoreactivity against monoclonal antibodies HP13 (thin line) and HP24 (thick line) by solid-phase enzyme-linked immunosorbent assay. Results are expressed as absorbance at 410 nm of the alkaline-phosphatase reaction product *p*-nitrophenol. Details of the procedures are given in the Experimental section. In (c) the fractions that reacted with monoclonal antibodies HP13 and HP24 were subjected to SDS/polyacrylamide-gel electrophoresis (50 μ l/lane except for the fraction that was eluted at 24 ml, from which 10 μ l was applied) and stained with AgNO_3 . The band designated by the arrow migrated at the M_r of purified human placenta PDI. The M_r markers (M) in lane 1, added at a concentration of 2 μ g of total protein, are as indicated for Fig. 1.

staining of a network that extended throughout the cell cytoplasm. No staining was observed when live HT-29 cells were incubated with monoclonal antibody HP24 (results not shown), precluding the presence of this particular epitope of PDI on the outer aspect of the

plasma membrane. Similar staining patterns were observed when live or fixed HT-29 cells were incubated with monoclonal antibodies HP13 or HP49. Antibody HP50 did not bind to either live or fixed HT-29 cells. Monoclonal antibody HP13 gave a similar staining

Fig. 5. Immunofluorescence microscopy of HT-29 human colon carcinoma cells with monoclonal antibody HP24

HT-29 cells were grown on glass slides, fixed with periodate/lysine/paraformaldehyde, and permeabilized with saponin to allow better access of antibodies to intracellular structures. Cells were then incubated with ascites fluid containing monoclonal antibody, followed by fluorescein-conjugated goat anti-(mouse IgG) antibody. (a) and (b) Immunofluorescence staining with monoclonal antibody HP24 and with an irrelevant antibody respectively; (c) and (d) phase-contrast photographs of the fields shown in (a) and (b) respectively. Note staining of the nuclear membrane and a network extending throughout the cytoplasm (a). Magnification $\times 573$.

pattern with IEC-18 normal rat small-intestinal epithelial cells (results not shown). The finding that antibodies raised against PDI from human placenta reacted with human and rat intestinal cells, as well as PDI from rat liver, suggests that there is no organ-specificity for this enzyme.

HT-29 cells were originally derived from secretory intestinal epithelium, and the finding of significant concentrations of immunoreactive PDI in these cells is consistent with the increased expression of PDI in secretory tissues (Chandler & Varandani, 1972; Freedman, 1984; Edman *et al.*, 1985). A major protein product of HT-29 cells is secretory component, the cell-surface receptor for dimeric immunoglobulin A (Huang *et al.*, 1976). Secretory component contains multiple intrachain disulphide bonds, one of which is reactive in the absence of protein denaturants and can form a disulphide linkage with the heavy chains of dimeric immunoglobulin A (Cunningham-Rundles & Lamm, 1975). Murkofsky & Lamm (1979) reported that the disulphide interchange reaction between secretory component and dimeric immunoglobulin A is promoted by an enzyme from rat liver microsomal fraction, a rich source of PDI (Mills *et al.*, 1983). Our monoclonal antibodies against human PDI should prove to be useful for studying a possible role of PDI in the interaction between secretory component and immunoglobulin A.

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