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In Vivo Cross-Linking of Protein Disulfide Isomerase to Immunoglobulins†

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ABSTRACT: To test the proposed role of protein disulfide isomerase in the synthesis of immunoglobulins (Ig), intact lymphocytes were treated with a thiol-cleavable, bifunctional cross-linking agent and lysed, and the lysates were immunoprecipitated with antibodies to either Ig or enzyme. When the immunoprecipitates were analyzed on polyacrylamide–sodium dodecyl sulfate gels, protein disulfide isomerase was found to be cross-linked to immunoglobulins. The extent of cross-linking was dependent upon the concentration of cross-linker added and the class of Ig. For IgMs and high concentrations of cross-linker, approximately one molecule of Ig was coupled per two molecules of enzyme. For IgGs, the extent of cross-linking was less. Finally, depletion of the intracellularly reduced glutathione by diamide was found to also result in the linkage of protein disulfide isomerase to IgM. These results therefore support the hypothesis that protein disulfide isomerase functions in the in vivo synthesis of immunoglobulins.

Protein disulfide isomerase (PDI;[1] EC 5.3.4.1) has been proposed to be involved in the formation of protein disulfide bonds in vivo [for reviews on this enzyme, see Morin and Dixon (1985) and Freedman (1984)]. A number of data are consistent with this hypothesis. First, the intracellular location of this enzyme on the luminal surface of the endoplasmic reticulum (Freedman, 1984) agrees with the proposed site for disulfide bond formation of proteins (Bergman & Kuehl, 1979). Second, the enzyme in vitro catalyzes the formation and breakage of disulfide bonds in a wide variety of proteins in a manner consistent with the in vivo synthesis of these proteins (Goldberger et al., 1963; Steiner et al., 1965; Corte & Parkhouse, 1973; Teale & Benjamin, 1976; Creighton et al., 1980; Koivu & Myllyla, 1986). Third, the levels of this enzyme (Brockway et al., 1980; Roth & Koshland, 1981; Myllyla et al., 1983) as well as its mRNA (Edman et al., 1985) in different cell types correlate with the extent of synthesis of disulfide-containing proteins in these cells.

However, PDI has also been proposed to have additional functions, for example, to participate in the degradation of insulin (Tomizawa & Halsey, 1959; Katzen & Stetten, 1962; Dawson & Varandani, 1987). Furthermore, other systems exist to promote the interchange of disulfide bonds, including thioredoxin (Holmgren, 1985; Pigiet & Schuster, 1986) and a cytoplasmic thiol–protein disulfide exchange activity (Axelsson et al., 1978). Thus, more direct evidence is required to establish a physiological role for PDI.

In the present studies we have sought to cross-link PDI with potential substrates for this enzyme in the intact cell. This in vivo cross-linking technique has the advantage that it allows one to analyze the native environment of the enzyme in the intact cell. Prior studies have utilized this approach to detect the clustering of the epidermal growth factor receptor (Fanger et al., 1986), oligomerization of the vesicular stomatitis viral glycoprotein (Kreis & Lodish, 1986), presence of additional subunits of the T-lymphocyte receptor (Brenner et al., 1985), and association of internalized insulin with a cytosolic metallothiol protease (Hari et al., 1987).

We have therefore utilized this approach to determine whether PDI can be cross-linked in vivo with one of its proposed substrates, immunoglobulin M (IgM). The immunoglobulin system was chosen since prior studies have demonstrated that PDI will, in vitro, promote the formation of the

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[1] Abbreviations: PDI, protein disulfide isomerase; Ig, immunoglobulin; DSP, dithiobis(succinimidyl propionate); PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; diamide, diazenedicarboxylic acid bis(*N*,*N*-dimethylamide); DTBP, dimethyl 3,3′-dithiobis(propionimidate) dihydrochloride; BSOCOES, bis[2-[[(succinimidooxy)carbonyl]oxy]ethyl] sulfone; GSH, glutathione; DMEM, Dulbecco's modified Eagle medium.

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interchain disulfide bonds required for monomer IgM assembly (Corte & Parkhouse, 1973; Roth & Koshland, 1981). Moreover, radioimmunoassays of lymphoid cells and their transformed counterparts indicated that the levels of PDI were 1–2 orders of magnitude higher in cells that are actively secreting immunoglobulins than those that are not (Roth & Koshland, 1981). The results presented in this paper document that PDI can be cross-linked with high efficiency to Ig in intact lymphocytes. These studies therefore support the proposed in vivo role for PDI in the assembly of Ig.

Experimental Procedures

Materials and Cell Lines. The predominant cell line used in these studies was a hybridoma producing an IgM monoclonal antibody (7D5) directed against the insulin receptor (Morgan & Roth, 1986). Additional cell lines tested were hybridomas producing IgM (25D4, 1C1), IgG (21D3, 28F2) (Morgan & Roth, 1986), and IgA (MOPC 315) (Eisen et al., 1968). These cells were grown in RPMI 1640 containing 10% fetal calf serum, 1 mM glutamine, 2 mM sodium pyruvate, 50 units/mL penicillin, 50 μg/mL streptomycin, and 50 μM mercaptoethanol.

The cross-linkers used in these studies, dithiobis(succinimidyl propionate), bis[2-[[(succinimidooxy)carbonyl]oxy]ethyl] sulfone, and dimethyl 3,3′-dithiobis(propionimidate) dihydrochloride, were from Pierce. Diamide was from Sigma.

Cross-Linking Protocol. Hybridomas ($\sim 10^6$ cells/mL) were metabolically labeled by culturing for 4–5 h in methionine- and cysteine-free DMEM containing 0.2 mCi/mL [^{35}S]methionine (trans ^{35}S label; ICN Radiochemicals, 600–1200 Ci/mmol). The cells were washed twice with ice-cold PBS (150 mM NaCl, 10 mM sodium phosphate, pH 7.4) and then incubated with PBS containing the indicated concentration of cross-linker for 30 min at 0 °C. The cells were then washed 2 more times and lysed with a buffer containing 1% Triton X-100, 1 mg/mL bacitracin, 20 mM EDTA, 20 mM Tris, and 20 mM Hepes, pH 7.5. After 30 min on ice, the lysates were centrifuged at 10000*g* for 10 min and the supernatants were utilized for immunoprecipitations. In the experiments utilizing diamide, cells were incubated with 10 mM diamide for 30 min at 37 °C and then processed as above without the addition of any cross-linker.

Immunoprecipitations. Lysates were first precleared with *Staphylococcus aureus* coated with control rabbit immunoglobulin (Sigma). Then the lysates were incubated with shaking overnight at 4 °C with the appropriate antibodies bound to *S. aureus*. To decrease binding of the mouse immunoglobulin to the *S. aureus*, control rabbit immunoglobulin (0.5 mg/mL) was added to the lysates. The antibody to PDI was an affinity-purified polyclonal rabbit antibody produced against PDI purified from rat liver (Roth, 1981). The antibody to mouse immunoglobulin was an affinity-purified polyclonal rabbit antibody (Pel Freeze).

The *S. aureus* complexes were washed 3 times in a buffer containing 1 M NaCl, 10 mM sodium phosphate, 0.02% azide, 0.1% SDS, 0.5% NP40, and 10 mg/mL bovine serum albumin. The precipitates were then washed once in the above buffer lacking albumin but containing 150 mM NaCl, transferred to fresh tubes, washed once with the above buffer containing 0.75 M LiCl, and then washed once with PBS. The samples were then brought to 1% in SDS and 5% mercaptoethanol and heated for 2 min at 100 °C, and the released proteins were electrophoresed on 12% polyacrylamide–SDS gels. Gels were stained, destained, soaked in Amplify (Amersham), dried, and exposed to film (Kodak XAR-5). To quantitate the extent of cross-linking, the Ig and PDI bands were excised and counted by liquid scintillation counting.

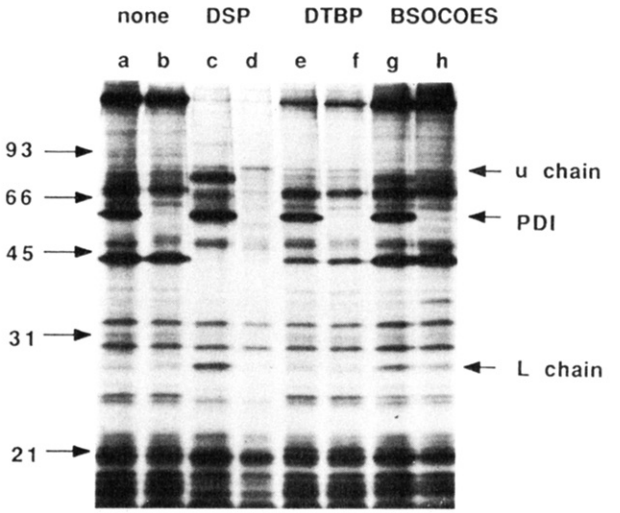

FIGURE 1: Cross-linking of PDI and IgM with different cross-linkers. Intact, metabolically labeled lymphocytes were incubated with either no cross-linker (a, b), 150 μg/mL DSP (c, d), 150 μg/mL DTBP (e, f), or 150 μg/mL BSOCOES (g, h). Cells were lysed, and the lysates were immunoprecipitated with either control immunoglobulin (b, d, f, h) or polyclonal anti-PDI antibodies (a, c, e, g). The immunoprecipitates were analyzed on 12% polyacrylamide–SDS gels after cleavage of the cross-linker. Molecular weights of protein standards ($\times 10^{-3}$) are shown. An autoradiogram of the gel is shown.

Immunoblots. For analyses by immunoblotting, electrophoresed samples were transferred to nitrocellulose filters for 3 h at 250 mA in a buffer containing 24 mM Tris base, 192 mM glycine, and 20% methanol. The filters were blocked by a 30-min incubation in Tris-buffered saline, pH 7.5, containing 3% bovine serum albumin and 0.1% Triton X-100. The filters were then incubated with either rabbit anti-mouse μ chain (1:250, Miles Laboratories) or a monoclonal antibody (Roth & Mesirow, 1984) to PDI (1:100 in Tris-buffered saline, 5% fetal calf sera, 0.05% Tween 20, and 0.5% bovine serum albumin). After a 16-h incubation at 4 °C, the filters were washed 4 times in Tris-buffered saline, pH 7.4, over a 1-h period at 24 °C. The bound immunoglobulin was then detected by use of alkaline phosphatase conjugated anti-mouse immunoglobulin (Promega Biotech) as detailed in the Promega handbook.

Results and Discussion

Cross-Linking of Ig and PDI by DSP. Metabolically labeled lymphocytes producing IgM were treated with various cleavable, bifunctional cross-linking agents. After 30 min at 4 °C, the cells were lysed and the lysates were immunoprecipitated with either antibodies to PDI or control immunoglobulin. The immunoprecipitates were extensively washed and then analyzed on polyacrylamide–SDS gels after cleavage of the cross-linkers. All the immunoprecipitates with antibodies to PDI, but not control Ig, contained a radioactive protein with an M_r of 60000, a value consistent with that reported for PDI (Carmichael et al., 1977) (Figure 1). In addition, immunoprecipitates from cells treated with the cross-linker DSP (Lomant & Fairbanks, 1976) contained radioactive proteins with M_r's of 75000 and 25000, values that are consistent with their identification as μ and light chains of Ig, respectively. These two proteins were not present in the control Ig immunoprecipitates from the same lysates of cross-linked cells.

Concentration Dependence of the Cross-Linking. Labeled lymphocytes were then incubated with various concentrations of DSP, lysed, and analyzed as described above. With in-

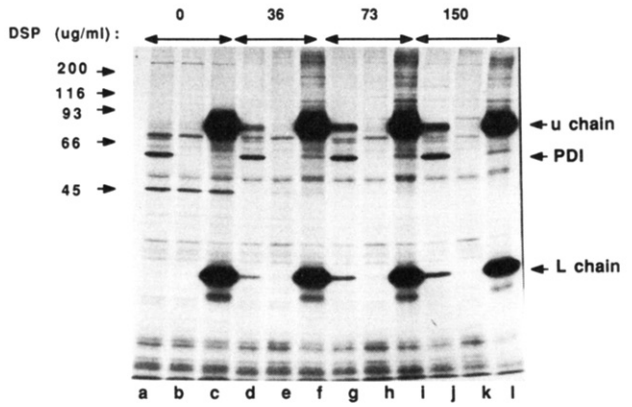

FIGURE 2: Cross-linking of PDI and IgM with different concentrations of cross-linker DSP. Intact, metabolically labeled lymphocytes were incubated with either no cross-linker (a–c), 36 μg/mL DSP (d–f), 73 μg/mL DSP (g–i), or 150 μg/mL DSP (j–l). Cells were lysed, and the lysates were immunoprecipitated with either anti-PDI antibodies (a, d, g, j), control immunoglobulin (b, e, h, k), or anti-mouse Ig (c, f, i, l). Immunoprecipitates were analyzed by SDS gel electrophoresis and autoradiography.

creasing concentrations of the cross-linker there were increasing amounts precipitated of the proteins with M_r's of 75000 and 25000 (Figure 2). In contrast, the amount of PDI precipitated remained constant. At higher concentrations of cross-linker, the amounts of PDI and even the nonspecifically precipitated proteins decreased (data not shown). This decrease probably occurs as a result of cross-linking of various proteins to the cytoskeletal proteins and their subsequent removal during centrifugation prior to immunoprecipitation.

When lysates of the same cross-linked cells were immunoprecipitated with antibodies to mouse Ig, a large amount of the labeled proteins with M_r's of 75000 and 25000 were precipitated (Figure 2). These results support the identification of these bands as the heavy and light chains of IgM. In addition, a protein of M_r 60000 could be observed in the immunoprecipitates with anti-Ig antibodies from lysates of cells treated with cross-linker but not of cells that were not cross-linked. Moreover, the amount of labeled protein of this molecular weight increased at the higher concentrations of cross-linker. The protein in this band was presumably PDI since it electrophoresed to the same position as this enzyme.

Detection of the Cross-Linked Proteins by Immunoblotting. To verify the identification of the cross-linked proteins of M_r 60000 and 75000, the immunoprecipitates from unlabeled cells were electrophoresed, transferred to nitrocellulose membranes, and probed with specific antibodies to either μ chain or PDI. In immunoprecipitates utilizing antibodies to PDI from cross-linked cells, a protein of M_r 75000 was detected with antibodies specific for μ chain (Figure 3). This protein was not detected in immunoprecipitates utilizing antibodies to PDI from lysates of cells that were not treated with cross-linker. Also, this protein was not detected in immunoprecipitates utilizing control Ig from lysates of cells that were treated with cross-linker.

Conversely, lysates were also immunoprecipitated with antibodies to Ig, and then the immunoprecipitates were electrophoresed, transferred to membranes, and probed with a monoclonal antibody to PDI (Roth & Mesirow, 1984). When lysates from cross-linked cells were utilized, a protein of M_r 60000 was detected (Figure 3). However, this protein was not detected in immunoprecipitates from lysates of cells that were not treated with cross-linker. These results therefore support the identification of the cross-linked proteins of M_r 75000 and 60000 as μ chain and PDI, respectively.

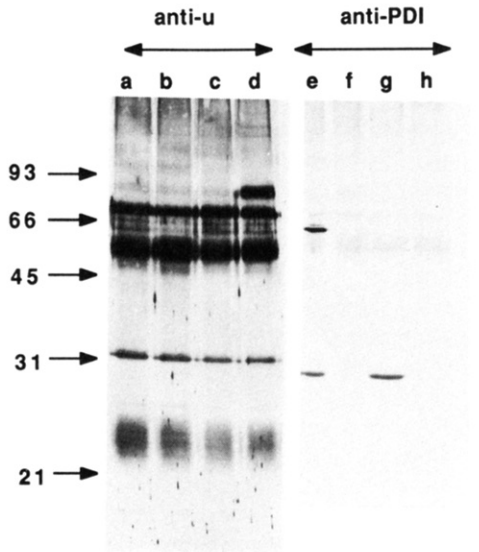

FIGURE 3: Analyses of cross-linked proteins by immunoblotting. Intact lymphocytes (nonlabeled) were treated with either no cross-linker (a, b, g, h) or 150 μg/mL DSP (c–f) and lysed, and the lysates were immunoprecipitated with either control immunoglobulin (a, c, f, h), antibodies to PDI (b, d), or mouse immunoglobulin (e, g). The immunoprecipitates were electrophoresed on 12% polyacrylamide–SDS gels and transferred to nitrocellulose filters, and the filters were probed with either polyclonal anti-μ chain (a–d) or monoclonal anti-PDI antibodies (e–h). The bands at 50 and 25 kDa present in lanes a–d represent rabbit Ig that is released from the immunoprecipitates and detected by the alkaline phosphatase conjugated anti-rabbit Ig utilized to develop these lanes.

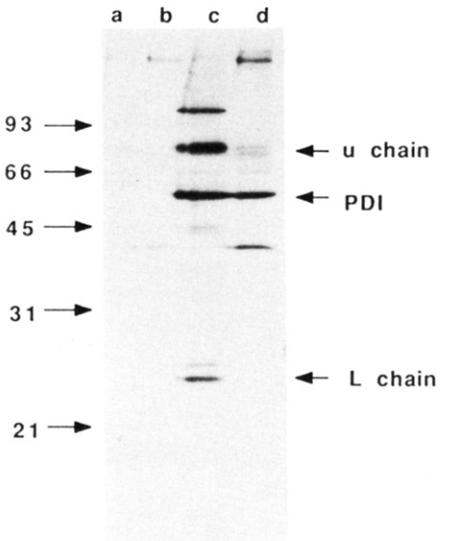

FIGURE 4: Linkage of PDI and IgM in cells depleted of glutathione. Intact, metabolically labeled lymphocytes were incubated with either buffer (b, d) or diamide (a, c) and lysed, and the lysates were immunoprecipitated with control Ig (a, b) or antibodies to PDI (c, d). The immunoprecipitates were analyzed by SDS gel electrophoresis and autoradiography.

Linkage of IgM to PDI in Cells Depleted of GSH. Since PDI in vitro forms a mixed disulfide complex with its substrates, which is subsequently released by GSH (Morin & Dixon, 1985; Freedman, 1984), it was possible that depletion of this metabolite would result in an accumulation of Ig linked to PDI via disulfide bonds. To test this hypothesis, we have utilized a thiol-oxidizing agent, diamide, which has been shown to oxidize GSH within human red blood cells (Kosower et al., 1969). Intact lymphocytes were treated with diamide and lysed, and the lysates were immunoprecipitated with antibodies to PDI. Immunoprecipitates from diamide-treated cells con-

tained, in addition to PDI, proteins that electrophoresed in positions identical with μ and light chains (Figure 4). In addition, a protein of $M_r \sim 110000$ was also detected. These labeled bands were not precipitated with control Ig from the same lysates of diamide-treated cells. Nor were they detected in immunoprecipitates utilizing antibodies to PDI from lysates of control cells. These data are consistent with the hypothesis that diamide decreased intracellular GSH levels and thereby resulted in a linkage of Ig to PDI. The extent of this linkage is comparable to that observed with the bifunctional cross-linker DSP (Figure 1).

Cross-Linking of PDI to Other Igs. To determine whether these results would be applicable to lymphocytes producing other Igs, five other cell lines were analyzed. Two of these lymphocyte lines produced other IgMs, two produced IgGs, and one produced IgA. In each case, antibodies to PDI were observed to precipitate immunoglobulins from lysates of cross-linked cells to a greater extent than control Ig. However, the extent of linkage of PDI to Ig appeared greater in cells producing μ chain than in those producing γ and α chains (data not shown). However, the cross-linking conditions were not optimized for the different Igs.

CONCLUSIONS

The present results demonstrate that the treatment of intact lymphocytes with a bifunctional, thiol-cleavable cross-linker (DSP) results in the cross-linking of PDI with immunoglobulins (Figure 1). Even at low concentrations of cross-linker, where nonspecific coupling is minimized, the cross-linking of PDI and IgM could be readily detected (Figure 2). At higher levels of cross-linker, the amount of μ chain linked to PDI increased and approached approximately one molecule of μ chain linked per two molecules of PDI.[2] This high level of cross-linking is also indicated by the finding that approximately one-third of the total cellular PDI was precipitated with anti-Ig antibodies from lysates of cross-linked cells (Figure 3 and unpublished data). Thus, the high efficiency of cross-linking as well as its detection at low concentrations of cross-linker indicates that PDI is in the vicinity of IgM in the native organization of the membrane and is involved in the synthesis of Ig. This hypothesis is further supported by the finding that the depletion of intracellular glutathione by diamide also resulted in the linkage of Ig and PDI by disulfide bonds. These latter results can be explained by the prior in vitro data which suggest that PDI forms a mixed disulfide complex with its substrates, which is subsequently released by GSH (Creighton et al., 1980; Carmichael et al., 1979). Finally, the present results also suggest that the approach of in vivo cross-linking could be utilized to identify substrates for other enzymes. For example, it might be possible to utilize this approach to identify substrates for receptors with intrinsic kinase activities.

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[2] The extent of linkage was calculated by determining the amount of radioactivity present in the μ and PDI bands precipitated by anti-PDI antibodies from cells treated with 150 μg/mL DSP. These calculations assume that the specific radioactivity of the methionine incorporated into the two proteins is the same and that there are 6–10 methionines in this particular μ chain (Kehry et al., 1979).