FEBS 18433 FEBS Letters 406 (1997) 291–295

Pancreas specific protein disulfide isomerase, PDIp, is in transient contact with secretory proteins during late stages of translocation

Jörg Volkmer[a], Silvia Guth[a], Wolfgang Nastainczyk[a], Peter Knippel[a], Peter Klappa[1,a], Volker Gnau[b], Richard Zimmermann[a,*]

[a] *Medizinische Biochemie, Universität des Saarlandes, D-66421 Homburg, Germany*
[b] *Applied Biosystems GmbH, Brunnenweg 13, D-64331 Weiterstadt, Germany*

Received 11 February 1997; revised version received 5 March 1997

Abstract Protein disulfide isomerase (PDI) and an additional lumenal protein of dog pancreas microsomes were previously observed to be in transient contact with secretory proteins during late stages of their co- or posttranslational translocation into these mammalian microsomes. The second protein was characterized as a 57 kDa glycoprotein. Here we identified this glycoprotein as the canine equivalent of human PDIp, a protein which was recently described as a new protein disulfide isomerase which is highly expressed in human pancreas. Canine PDIp is also a very abundant protein, its concentration in pancreatic microsomes approaches the concentration of PDI and of the major microsomal molecular chaperones. Apparently, PDIp shares with PDI not just the enzymatic but also the polypeptide binding or chaperoning activity. Furthermore, we suggest that PDIp, too, can be involved in completion of cotranslational as well as posttranslational translocation of proteins into mammalian microsomes.

Key words: Protein disulfide isomerase; Molecular chaperone; Protein transport; Endoplasmic reticulum; Secretory protein

1. Introduction

A major question in cell biology is concerned with the mechanisms of intracellular protein transport. Several laboratories have studied the mechanisms of cotranslational and posttranslational protein transport into mammalian microsomes by different crosslinking approaches. With respect to cotranslational transport, it was observed that translation of truncated mRNAs related to bovine preprolactin mRNA in the presence of dog pancreas microsomes leads to the signal recognition particle (SRP)/docking protein and GTP dependent membrane association of ribosomes, which contain nascent preprolactin chains as peptidyl-tRNAs [1–6]. Furthermore, it was found that nascent chains, such as 86-residue preprolactin (comprising the 86 amino-terminal amino acid residues of preprolactin), become associated with the microsomal membrane proteins Sec61α and TRAM protein under these conditions [7–14]. Following their release from Sec61α and TRAM protein, the corresponding mature polypeptide chains, such as 56-residue prolactin (comprising the 56 amino-terminal amino acid residues of prolactin), were found to transiently associate with protein disulfide isomerase (PDI) and a 57 kDa glycoprotein [15–17]. Similar observations with respect to membrane proteins and lumenal proteins were made for posttranslational transport [15,16]. Since mature polypeptides which lacked cysteine residues were also found transiently associated with PDI [15,16] we suggested that PDI may play a role in completion of cotranslational and posttranslational translocation [16] and that this activity of PDI may be related to its general chaperoning activity [18–20]. Here we identified the 57 kDa glycoprotein as the canine equivalent of human protein disulfide isomerase p (PDIp) [21]. Therefore, we propose that PDIp and PDI may have overlapping functions not only in disulfide formation but also in protein transport and protein folding.

2. Materials and methods

2.1. Materials

L-[^{35}S]Methionine was obtained from Amersham. *Pvu*II, *Sca*I, proteinase K, endoglyosidase H (Endo H) and SP6 polymerase were purchased from Boehringer Mannheim. X-ray films (X-Omat AR) were from Kodak. Dithiobis(succinimidylpropionate) (DSP), dimethyl suberimidate and succinimidyl 4-(*N*-maleimidomethyl)cyclohexanecarboxylate (SMCC) were obtained from Pierce Chemical Co. Puromycin was from Sigma. Phenylmethylsulfonyl fluoride was purchased from Merck. Antibodies directed against bovine liver protein disulfide isomerase (SPA-890), chicken oviduct Grp94 (SPA-850), and a C-terminal peptide of rat Grp78 (SPA-826) were from StressGen. Protein A-Sepharose was purchased from Pharmacia Biotech. 3-[(3-Cholroamidopropyl)-dimethylamino]-propanesulfonate (CHAPS) was obtained from Calbiochem. Hydroxyapatite was from Biorad.

2.2. In vitro translation and transport

In vitro translation and transport were carried out as described previously [15,16]. Plasmid pB4 which contains the preprolactin coding region behind the SP6 promoter was linearized with *Pvu*II (giving 86-residue preprolactin as a translation product) and transcribed as described [16]. Plasmid pCA2 coding for a hybrid between preprocecropin A and the cytosolic protein dihydrofolate reductase was linearized with *Sca*I within the coding region (giving 98-residue ppcecDHFR) and transcribed as described [15]. Crosslinking with SMCC and DSP, respectively, was carried out as described [15,16].

2.3. Purification of PDIp from dog pancreas microsomes

Dog pancreas microsomes were isolated as described previously [15]. They were reisolated by centrifugation and subjected to detergent solubilization at low ionic strength (0.65% CHAPS, 200 mM KCl, 2 mM EDTA, 1.5 mM $MgCl_2$, 15% (w/v) glycerol, 20 mM HEPES/KOH pH 7.5). The solubilized proteins (i.e. lumenal proteins) were separated from the insoluble material (i.e. membrane proteins) by centrifugation. 10 ml of the solubilized proteins was subjected to chromatography on hydroxyapatite (10 ml bed volume) which had been equilibrated with the solubilization buffer. After washing the column with three bed volumes of solubilization buffer the bound proteins were eluted with a phosphate gradient (made from 40 ml of low-salt solubilization buffer containing between 0 and 100 mM

*Corresponding author. Fax: (49) (6841) 166288.

[1] *Present address:* Biological Laboratory, The University of Kent, Canterbury, Kent CT2 7NJ, UK.

PII S0014-5793(97)00288-3

potassium phosphate). The fractions which were highly enriched in a 57 kDa glycoprotein (i.e. a 57 kDa protein which was sensitive towards Endo H treatment) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After staining of the gel the major band was cut and subjected to electroelution. The eluted protein was subjected to two-dimensional gel electrophoresis for analysis of its purity and used for the production of rabbit antibodies and for sequence analysis.

The PDI activity assay was carried out according to Holmgren [22,23]. For immunodepletion experiments the antibodies were coupled to protein A-Sepharose by chemical crosslinking with dimethyl suberimidate.

2.4. Analytical methods

Immunoprecipitations with the respective antisera and protein A-Sepharose were performed in high-salt solubilization buffer (0.5% CHAPS, 400 mM KCl, 1 mM EDTA, 1.5 mM $MgCl_2$, 30% (w/v) glycerol, 20 mM HEPES/KOH pH 7.5) but otherwise as described previously [16]. For one-dimensional separation of proteins, samples were subjected to electrophoresis in high Tris/urea/sodium dodecyl sulfate-polyacrylamide gels and subsequent fluorography as described [15,16]. Two-dimensional gel electrophoresis was carried out according to published procedures [24]. BrCN cleavage and Edman degradation of cleavage products was carried out as described previously [25].

A

```
1   MASCPWGQEQGARSPSEEPPEEEIPKEDGILVLSRHTLGL
41  ALREHPALLVEFYAPWCGHCQALAPEYSKAAAVLAAESMV
81  VTLAKVDGPAQRELAEEFGVTEYPTLKFFRNGNRTHPEEY
121 TGPRDAEGIAEWLRRRVGPSAMRLEDEAAAQALIGGRDLV
                    MRLAKKEAAQALIDLRDAA
161 VIGFFQDLQDEDVATFLALAQDALDMTFGLTDRPRLFQQF
    VIGFFRARLDEQV
201 GLTKDTVVLFKKFDEGRADFPVDEELGLDLGDLSRFLVTH
241 SMRLVTEFNSQTSAKIFAARILNHLLLFVNQTLAAHRELL
    MHLVTEFNSQTSPKIFAARILNHLLLFVQQTLAEHRELL
281 AGFGEAAPRFRGQVLFVVVDVAADNEHVLQYFGLKAEAAP
    EGFGEAAP
321 TLRLVNLETTKKYAPVDGGPVTAASITAFCHAVLNGQVKP
361 YLLSQEIPPDWDQRPVKTLVGKNFEQVAFDETKNVFVKFY
401 APWCTHCKEMAPAWEALAEKYQDHEDIIIAELDATANELD
                MAAAWEALAEKYKDHEDIVIAELDATANELE
441 AFAVHGFPTLKYFPAGPGRKVIEYKSTRDLETFSKFLDNG
    AFP
481 GVLPTEESPEEPAAPFPEPPANSTMGSKEEL
```

B

	PDI	Grp58	CaBP2	P5	PDIR	PDIp
Peptide 1	31	28	16	0	0	59
Peptide 2	40	4	13	9	9	89
Peptide 3	50	35	38	3	21	82

Fig. 1. Sequence comparison for human PDIp and three BrCN cleavage products which were derived from a canine microsomal 57 kDa glycoprotein. A methionine residue was added to the amino-termini of the sequences of the three BrCN cleavage products on the assumption that endogenous peptides were subjected to sequencing. The comparison was carried out following the Clustal method, provided by the DNASTAR sequence analysis software. A: Identical amino acid residues are indicated by shaded boxes. There were two thioredoxin-like catalytic sites (56–61 and 403–408), an ER retention sequence and two potential *N*-linked glycosylation sites (113–115 and 502–504) detected in the human protein [21]. B: The contents of identical amino acid residues are indicated (%). PDI (ERp59), Grp58 (ERp57, ERp61, ER-60), CaBP2 (ERp72), P5 (CaBP1) and PDIR are also known as the proteins which are given in parentheses and that the data are given for mouse PDI, bovine Grp58, rat CaBP2 and human P5, PDIP and PDIp.

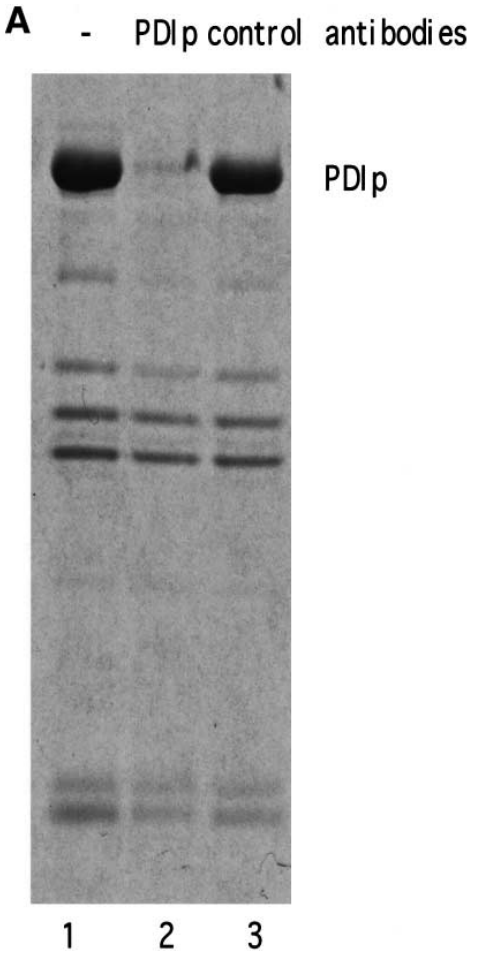
Fig. 2. PDI activity assay for the canine microsomal 57 kDa glycoprotein. A crude PDIp containing fraction which was obtained by chromatography of lumenal proteins on hydroxyapatite (control, open squares in B) as well as two derivatives of this fraction were subjected to SDS-PAGE which was followed by protein staining with Coomassie brilliant blue (A) and to PDI activity assays (B). The two derivatives of the control fraction were obtained by immunodepletion either with protein A-Sepharose (−anti-PDIp, filled squares in B) or with anti-PDIp bound to protein A-Sepharose (+anti-PDIp, open circles in B). The PDI activity was measured as reductive cleavage of insulin and accompanying formation of B chain precipitates according to published procedures [22,23] and is given for the three PDIp samples and a negative control which did not contain any enzyme (filled triangles).

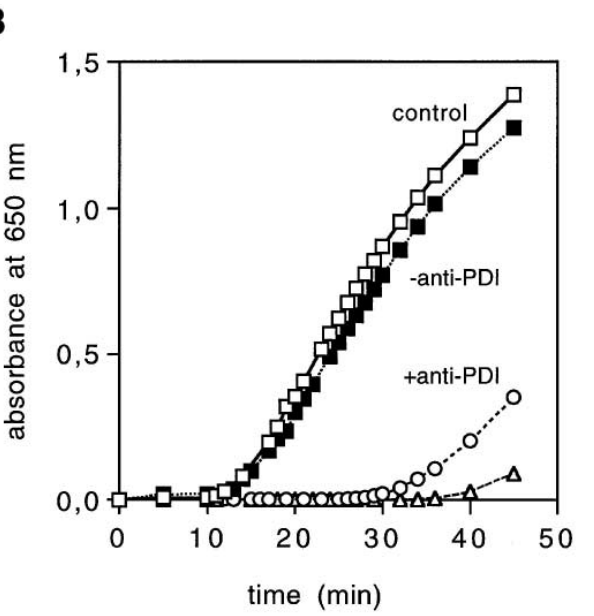
→

3. Results

3.1. PDIp is an abundant protein in dog pancreas microsomes

A 57 kDa protein of dog pancreas microsomes was previously found in association with secretory proteins during late stages of their membrane transport. This protein was shown to be a lumenal glycoprotein. In an attempt to identify this protein, lumenal proteins were fractionated by chromatography on hydroxyapatite. The fractions were analyzed by SDS-PAGE. Fractions containing a single protein as major component were pooled. The enriched proteins were subjected to Endo H treatment. On the basis of its molecular mass and its Endo H sensitivity a protein was selected for immunization. SDS-PAGE was carried out on a preparative scale. The 57 kDa glycoprotein was visualized by protein staining and eluted from the respective gel slice by electro-elution. The eluted protein which was pure according to two-dimensional gel electrophoresis was used for immunization. In parallel, a corresponding gel slice was dried in vacuo and subjected to in situ BrCN cleavage [25]. The cleavage products were separated by SDS-PAGE and transferred to PVDF membranes. The cleavage products were visualized by protein staining and the three major cleavage products were subjected to Edman degradation. The data from the sequence analysis and the subsequent database search led to the sequence comparisons which are shown in Fig. 1. The BrCN products obtained from the canine microsomal 57 kDa glycoprotein showed the highest level of identity to human PDIp, a recently identified member of the family of protein disulfide isomerases which is highly expressed in human pancreas [21]. Therefore, we asked if the canine microsomal 57 kDa glycoprotein has PDI activity. The enriched protein fraction, obtained by chromatography of lumenal proteins on hydroxyapatite, was subjected to an established PDI activity assay [22,23]. In addition to the crude fraction (Fig. 2A, lane 1), two samples were assayed. One sample had been derived from the crude fraction by incubation with PDIp antibodies, bound to protein A-Sepharose (Fig. 2A, lane 2), the other sample by incubation with protein A-Sepharose (Fig. 2A, lane 3). The crude fraction





contained PDI activity which could be depleted from the fraction by incubation with protein A-bound anti-PDIp antibodies but not with protein A-Sepharose (Fig. 2B).

Thus the purified canine microsomal 57 kDa glycoprotein represents the canine equivalent to human PDIp. Canine PDIp is one of the major lumenal proteins of dog pancreas microsomes according to two-dimensional gel electrophoresis (Fig. 3).

3.2. *PDIp is crosslinked to mature secretory proteins*

Next we asked whether the antibodies which are directed against canine PDIp recognize the crosslinking products which had been observed for the microsomal 57 kDa glycoprotein and various mature secretory proteins during late stages of their membrane transport [15,16]. The mature proteins which had been analyzed previously either contained (56-residue prolactin, 76-residue procecropin DHFR) or did not contain (47-residue procecropin A, procecropin A) cysteine residues and had been found to be transported co- (56-residue prolactin) or posttranslationally (47-residue procecropin A, procecropin A, 76-residue procecropin DHFR) [15,16]. Furthermore, we had observed that the mature proteins differed in their efficiencies of binding and/or crosslinking to PDI and the 57 kDa glycoprotein. In the case of 56-residue prolactin and procecropin A the dominant crosslinking products in the relevant molecular mass range comprised PDI [16] (also see Fig. 4A, lane 1), in the case of 76-residue procecropin DHFR the dominant crosslinking product in the relevant molecular

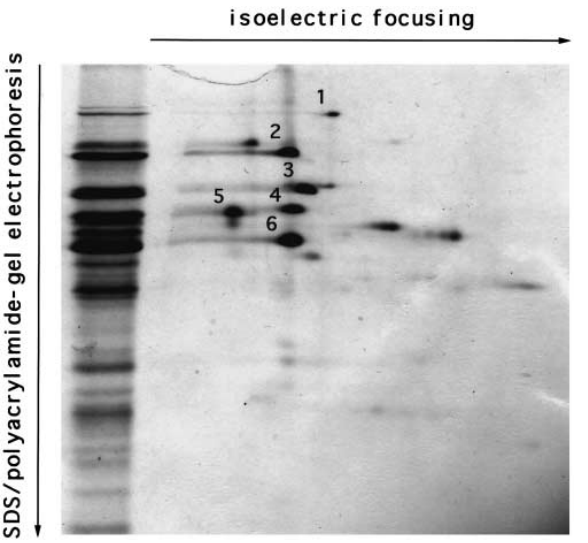
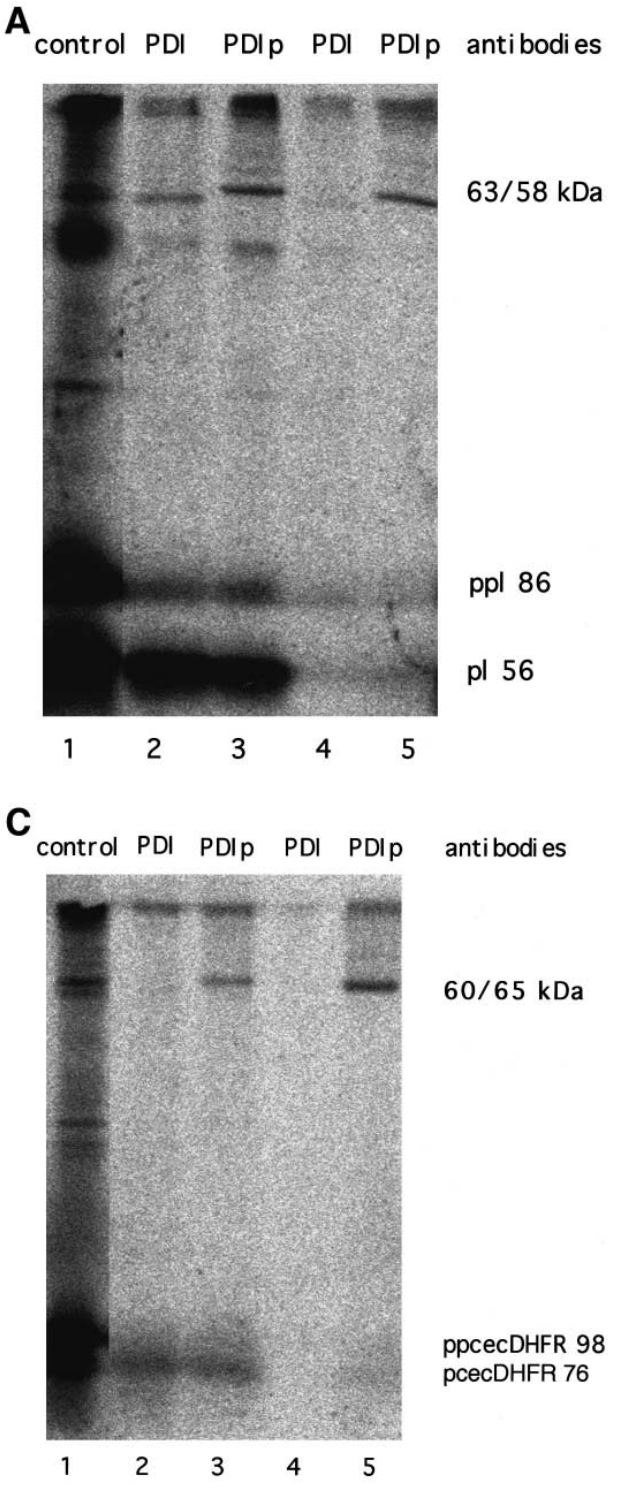
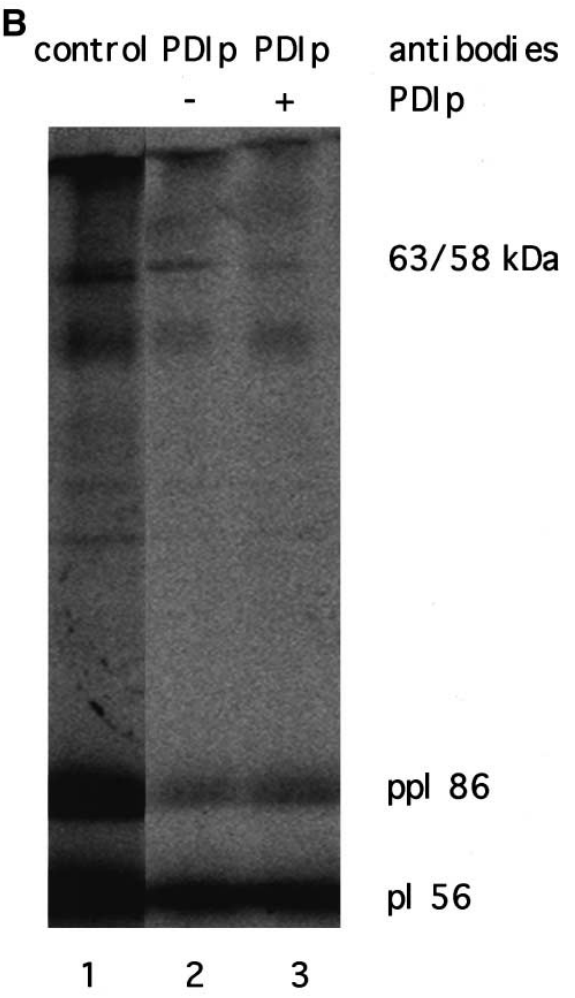


Fig. 3. Two-dimensional gel electrophoresis of lumenal proteins from dog pancreas microsomes. The lumenal proteins were extracted from dog pancreas microsomes as described in Section 2. Subsequently they were subjected either to SDS-PAGE (lane on the left) or to two-dimensional gel electrophoresis, comprising isoelectric focusing as the first dimension and SDS PAGE as the second dimension. The major spots, which were numbered 1 (Grp170), 2 (Grp94), 3 (Grp78, BiP), 4 (PDIp), 5 (calreticulin, CaBP3, ERp60), and 6 (PDI, ERp59), were identified by Western blotting and decoration with specific antibodies as the proteins which are indicated in parentheses.





mass range comprised the 57 kDa glycoprotein [15] (see also Fig. 4C, lane 1).

Here, 86-residue preprolactin or 98-residue preprocecropin DHFR was transported into dog pancreas microsomes under co- or posttranslational conditions. Crosslinking and immunoprecipitation were carried out in order to identify the 57 kDa microsomal protein giving rise to the 63 kDa crosslinking product in the case of 56-residue prolactin. The 63 kDa crosslinking product was immunoprecipitated by antibodies directed against canine PDIp (Fig. 4A,B). The corresponding 65 kDa crosslinking product observed with 76-residue procecropin DHFR was immunoprecipitated with antibodies directed against PDIp (Fig. 4C), too.

Thus PDIp binds to different secretory proteins after their translocation into the endoplasmic reticulum. Taken together with our previous observations [15,16], PDIp also interacts with proteins which do not contain cysteine residues.

Fig. 4. Immunoprecipitation of the crosslinking products. A, B: 86-residue preprolactin was synthesized by in vitro translation in the presence of [^{35}S]methionine in the presence of dog pancreas microsomes for 20 min. The translation products were reisolated by centrifugation and resuspended in SP buffer [16]. Then the samples were supplied with puromycin and incubated at 30°C for 10 min. The incubation was terminated by freezing the samples in liquid nitrogen. Subsequently, the samples were supplied with the same volume of XL solution A (670 μM SMCC in SP buffer). C: 98-residue preprocecropin DHFR was synthesized in vitro in the presence of [^{35}S]methionine for 20 min. The translation reaction was supplied with puromycin and incubated at 30°C for 5 min. Subsequently, dog pancreas microsomes were added and the incubation was continued for 15 min. The incubation was terminated by freezing the samples in liquid nitrogen. After thawing on ice, the translation products were reisolated by centrifugation and resuspended in SP buffer. Then the samples were supplied with the same volume of XL solution B (296 μM DSP in SP buffer) [16]. A, B, C: All crosslinking products were reisolated by centrifugation and subjected to immunoprecipitation with antibodies directed against PDIp or PDI (StressGen, SPA-890) in high salt solubilization buffer (see Section 2). The immunoprecipitates were analyzed by gel electrophoresis and fluorography. We note that for the immunoprecipitations four equivalents of the respective control samples were employed. A, C: Triton X-100 was added to the immunoprecipitation reactions which are presented in lanes 4 and 5 (final concentration: 1%). B: For the competition experiment antibodies against PDIp were preincubated for 10 min with either buffer (15 mM NH_4HCO_3, pH 8.5) or purified PDIp in buffer (20 μg). We note that the signal which was obtained for 98-residue preprocecropin DHFR and anti-PDI antibodies (shown in C, lanes 2 and 4) was very weak due to the low representation of the respective crosslinking product in the material which was subjected to immunoprecipitation (i.e. as compared to 86-residue preprolactin) and to the low efficiency of the anti-PDI antibodies (i.e. as compared to the anti-PDIp antibodies).
←

4. Discussion

Previously we had found various mature secretory proteins in transient contact with a 57 kDa glycoprotein and a 52 kDa nonglycosylated microsomal protein after they had been released from the protein translocase in the microsomal membrane [15,16]. The 52 kDa protein had been identified as PDI [16]. Here the crosslinking product comprising the 57 kDa lumenal protein was immunoprecipitated by antibodies directed against PDIp. This protein has been shown to be a catalyst of disulfide bond formation [21]. Since there are two cysteine residues present in 56-residue prolactin it was not unexpected to find PDIp interacting with this protein. In the case of various procecropin derivatives, however, there are no cysteine residues present [15,16]. Nevertheless PDIp was found to interact with these mature proteins. We suggest that in the case of preprocecropin derivatives PDIp is not involved in the formation of disulfide bonds, but probably has a chaperoning function. Therefore, we propose that PDIp and PDI may represent two of the reticuloplasmic proteins which were shown to be involved in completion of translocation [26], i.e. binding of a secretory protein to either one of these lumenal chaperones may be involved in efficient unidirectional translocation.

Acknowledgements: We thank Dr. H.-D. Söling (Göttingen) for the kind gift of antibodies directed against calreticulin. This work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

References

[1] T. Connolly, R. Gilmore, J Cell Biol 103 (1986) 2253–2261.
[2] T.V. Kurzchalia, M. Wiedmann, A.S. Girshovich, E.S. Bochkareva, H. Bielka, T.A. Rapoport, Nature 320 (1996) 634–636.
[3] U.C. Krieg, P. Walter, A.E. Johnson, Proc Natl Acad Sci USA 83 (1986) 8604–8608.
[4] T. Connolly, R. Gilmore, Cell 57 (1989) 599–610.
[5] J.D. Miller, H. Wilhelm, L. Gierasch, R. Gilmore, P. Walter, Nature 366 (1993) 351–354.
[6] G. Bacher, H. Lütcke, B. Jungnickel, T.A. Rapoport, B. Dobberstein, Nature 381 (1996) 248–251.
[7] M. Wiedmann, T.V. Kurzchalia, E. Hartmann, T.A. Rapoport, Nature 328 (1987) 830–833.
[8] U.C. Krieg, A.E. Johnson, P. Walter, J Cell Biol 109 (1989) 2033–2043.
[9] R.N. Thrift, D.W. Andrews, P. Walter, A.E. Johnson, J Cell Biol 112 (1991) 809–821.
[10] S. High, D. Görlich, M. Wiedmann, T.A. Rapoport, B. Dobberstein, J Cell Biol 113 (1991) 35–44.
[11] S. High, S.L. Andersen, D. Görlich, E. Hartmann, S. Prehn, T.A. Rapoport, B. Dobberstein, J Cell Biol 121 (1993) 743–750.
[12] D. Görlich, E. Hartmann, S. Prehn, T.A. Rapoport, Nature 357 (1992) 47–52.
[13] D. Görlich, S. Prehn, E. Hartmann, K.-U. Kalies, T.A. Rapoport, Cell 71 (1992) 489–503.
[14] E. Hartmann, T. Sommer, S. Prehn, D. Görlich, S. Jentsch, T.A. Rapoport, Nature 367 (1994) 654–657.
[15] P. Klappa, M. Zimmermann, R. Zimmermann, FEBS Lett 341 (1994) 281–287.
[16] P. Klappa, R. Freedman, R. Zimmermann, Eur J Biochem 232 (1995) 755–764.
[17] J.D. Oliver, F.J. van der Wal, N.J. Bulleid, S. High, Science 275 (1997) 86–88.
[18] R. Noiva, H. Kimura, J. Roos, W.J. Lennarz, J Biol Chem 266 (1991) 19645–19649.
[19] M.L. LaMantia, W.J. Lennarz, Cell 74 (1993) 899–908.
[20] A. Puig, H.F. Gilbert, J Biol Chem 269 (1994) 7764–7771.
[21] M.G. Desilva, J. Lu, G. Donadel, W.S. Modi, H. Xie, A.L. Notkins, M.S. Lan, DNA Cell Biol 15 (1996) 9–16.
[22] A. Holmgren, J Biol Chem 254 (1979) 9627–9632.
[23] P.N. Van, K. Rupp, A. Lampen, H.-D. Söling, Eur J Biochem 213 (1993) 789–795.
[24] H. Wiech, J. Buchner, M. Zimmermann, R. Zimmermann, U. Jakob, J Biol Chem 268 (1993) 7414–7421.
[25] T. Dierks, J. Volkmer, G. Schlenstedt, C. Jung, U. Sandholzer, K. Zachmann, P. Schlotterhose, K. Neifer, B. Schmidt, R. Zimmermann, EMBO J 15 (1996) 6931–6942.
[26] C.V. Nicchitta, G. Blobel, Cell 73 (1993) 989–998.