Journal of Cellular Biochemistry 68:436–445 (1998)

Elevated Expression of PDI Family Proteins During Differentiation of Mouse F9 Teratocarcinoma Cells

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Abstract We investigated the expression of protein disulfide isomerase family proteins (PDI, ERp61, and ERp72) in mouse F9 teratocarcinoma cells during differentiation induced by treatment with retinoic acid and dibutyryl cAMP. Each member of this family was expressed at a constitutive level in undifferentiated F9 cells. During differentiation of F9 cells to parietal or visceral endodermal cells the protein level of all these enzymes increased, although the extent of this increase in both protein and mRNA levels varied among the enzymes. Certain proteins were found to be co-immunoprecipitated with PDI, ERp61, and ERp72 in the presence of a chemical crosslinker. Type IV collagen was significantly coprecipitated with PDI whereas laminin was equally coprecipitated with the three proteins. Furthermore, 210 kDa protein characteristically coprecipitated with ERp72. Thus, the induction of PDI family proteins during the differentiation of F9 cells and their association with different proteins may implicate specific functions of each member of this family despite the common redox activity capable of catalyzing the disulfide bond formation. J. Cell. Biochem. 68:436–445, 1998.

Key words: mouse; PDI family proteins; retinoic acid; dibutyryl cAMP; differentiation

INTRODUCTION

Although the final conformation of proteins is determined by its amino acid sequence, the folding and assembly of newly synthesized proteins in vivo involve two classes of proteins. One is a group of molecular chaperones that bind to unfolded or partially folded polypeptides and prevent adoption of an inappropriate conformation. The other class of proteins is enzymes that catalyze the formation or isomerization of intra- or intermolecular covalent bonds such as disulfide bonds and cis/trans prolyl isomerization in newly synthesized proteins [Lang and Schmid, 1988; Gething and Sambrook, 1992]. The best characterized of the latter is protein disulfide isomerase (PDI) that forms disulfide bonds inbetween polypeptide(s) [Bulleid and Freedman, 1988; Bardwell and Beckwith, 1993; LaMantia and Lennarz, 1993; Weissman and Kim, 1993]. The PDI molecule includes a duplicate of a characteristic domain that shows high homology to thioredoxin, a small cytoplasmic redox protein present in all classes of organisms, containing a tetrapeptide of Cys-Gly-His-Cys (CGHC) motif as an active site involved in thiol:protein oxidoreductase activity. PDI also possesses tetrapeptide of Lys-Asp-Glu-Leu (KDEL) in its COOH-terminus which functions as an endoplasmic reticulum (ER) retention signal playing an important role in respect to catalysis of newly synthesized protein in ER [Edman et al; 1985; Munro and Pelham, 1987].

Other proteins containing thioredoxin-like domain and ER retention signal have been described to form a distinct protein family [Bennett et al., 1988; Mazzarella et al., 1990; Chaudhuri et al., 1992]. ERp61 (ER60, Q2, GRP58) and ERp72 (CaBP2) are members of

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Received 25 August 1997; Accepted 10 October 1997

this family and both were proved to possess thiol:protein oxidoreductase activity as well as PDI [Lewis et al., 1985a,b; 1986; Bennett et al., 1988; Mazzarella et al., 1990, 1994; Srivastava et al., 1991, 1993; Urade et al., 1992; Kozaki et al., 1994; Iida et al., 1996]. ERp61 and ERp72 contain two and three thioredoxin-like domains, respectively, and there are tetrapeptides of Gin-Glu-Asp-Leu (QEDL) and Lys-Glu-Glu-Leu (KEEL) in the COOH termini of ERp61 and ERp72, respectively, which are regarded as variants of the consensus ER-retention signal (KDEL) present in PDI [Bennett et al., 1988; Mazzarella et al., 1990]. Although the precise biological role of each enzyme is still unknown, the presence of common active sites responsible for redox activity and the actual thiol:protein oxidoreductase activity of ERp61 and ERp72 suggests that these proteins are also able to form disulfide bonds of nascent polypeptides in the ER as well as PDI. Our previous immunohistochemical studies of PDI family proteins in the mouse and rat tissues showed the characteristic distributions of these proteins in certain cell types, supposing the presence of substrate preference for the different enzymes [Kozaki et al., 1994; Iida et al., 1996].

F9 stem cells are induced to differentiate into a cell type indistinguishable from definitive parietal or visceral endodermal cells in the presence of retinoic acid and dibutyryl cyclic AMP (cAMP) in the monolayer culture or retinoic acid alone in suspension culture, respectively [Strickland and Mahdavi, 1978, Strickland et al., 1980; Hogan et al., 1981; Wang et al., 1985; Mason et al., 1986]. The differentiation into parietal endodermal cells subsequently causes marked alteration in the expression levels of various secretary proteins including tissue plasminogen activator, type IV collagen, prolyl 4-hydroxylase, laminin, SPARC (secreted, acidic, cysteine-rich glycoprotein), and collagen-binding stress protein, HSP47 [Strickland et al., 1980; Kurkinen et al., 1984; Wang et al., 1985; Mason et al., 1986; Takechi et al., 1992].

In the present study, we investigated the expression of PDI family proteins in mouse F9 teratocarcinoma cells during differentiation. The different degree of PDI, ERp61, and ERp72 induction and the association of different protein(s) might suggest the individual roles of PDI, ERp61, and ERp72.

MATERIALS AND METHODS

Chemical Reagents and Antibodies

The chemical crosslinkers dithio*bis* (succinimidylpropionate) (DSP) and disuccinimidyl suberate (DSS) were purchased from Nacalai Tesque, Inc. (Kyoto Japan). 3,3′-dithio*bis*(sulfosuccinimidylpropionate) (DTSSP) was from Pierce Chemical Co. (Rockford, IL). Chromatographically purified bacterial collagenase (Form III) was obtained from Advance Biofactures Co. (Lynbrook, NY). Affinity-purified rabbit polyclonal antibody against ERp61 [Kozaki et al., 1994] and ERp72 [Iida et al., 1996] has been described elsewhere. Rabbit polyclonal antibody against PDI was prepared and affinity-purified by the same method as ERp72 [Iida et al., 1996] using bovine PDI (Boehringer, Germany), as antigen. Rabbit polyclonal antibody against laminin was a kind gift from Dr. Kimata (Institute of Molecular Medicine, Aichi Medical University). Goat polyclonal antibody against type IV collagen was purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL). Affinity-purified FITC-conjugated goat anti-rabbit IgG was obtained from Tago (Burlingame, CA).

Cells and Cell Culture

Mouse F9 teratocarcinoma cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum. For differentiation, the medium containing 2.5 μM retinoic acid (Sigma, St. Louis, MO) and 2.5 mM dibutyryl cAMP (Behringer, Germany) was used and exchanged daily. Cells cultured in this medium for 96 h were used in all experiments.

Immunofluorescence

Cells cultured on coverslips were used for immunofluorescence. F9 cells and differentiated F9 cells (dF9 cells) were fixed in 0.1 M sodium phosphate buffer, pH 7.2 containing 12.5% formalin for 1 h. After rinsing with PBS containing 0.1% glycine, cells were permeabilized by 0.1% Triton X-100 in PBS for 4 min, followed by immunofluorescent staining as described previously [Saga et al., 1987]. Affinity-purified rabbit antibody against PDI, ERp61, or ERp72 was used for the first antibody, and affinity-purified FITC-conjugated anti-rabbit IgG for the second antibody. All immunofluorescence specimens were observed under a confocal laser

scanning microscope equipped with a Krypton-Argon laser (MRC600, Bio-Rad, Watford, UK).

Crosslinking and Immunoprecipitation

Cultured F9 cells were labeled in the medium supplemented 1.85 MBq/ml Tran^{35}S-label (ICN, Irvine CA) for 12 h. After rinsing with Ca^{2+}- and Mg^{2+}-free PBS three times, the cells were scraped and resuspended in PBS, then combined with 2% of 0.1 M DSP (in DMSO), vortexed and placed on ice for 30 min. In some experiments, DSS (0.1 M in DMSO) and DTSSP (0.1 M in distilled water) were used in place of DSP. After 30 min, the cells were rinsed with PBS containing 2 mM glycine and lysed in a lysis buffer (2% NP40, 50 mM Tris-HCI, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1 mM *N*-ethylmaleimide, 1 mM PMSF, 1 µg/ml leupeptin, and 1 µg/ml pepstatin A) followed by sonication for 10 s. Cell lysates were obtained by centrifugation at 12,000*g* for 10 min, and used for immunoprecipitation. Equal counts of cell lysate were incubated with anti-PDI, ERp61, or ERp72 polyclonal antibody in a buffer containing 1% NP40, 50 mM Tris-HCl pH 8.0 buffer, containing 150 mM NaCl, 5 mM EDTA for 1 h at 4°C, and then added protein G conjugated Sepharose FF (Pharmacia, Piscataway, NJ) and incubated in the same condition. After rinsing three times with the same buffer, immunoprecipitates were eluted in SDS-sample buffer by boiling and served for one-dimensional SDS-PAGE. Densitometric analysis was performed using NIH-image programme on an Apple computer system.

Collagenase Treatment

Immune complexes precipitated with protein G Sepharose (20 µl bed volume) were suspended in 40 ml of collagenase buffer (50 mM Tris-HCl, pH 7.3, 2 mM *N*-ethylmaleimide, 10 mM $CaCl_2$) containing 20 U/ml collagenase, and incubated at 37°C for 60 min. The samples were then boiled for 5 min in 15 ml of 5 × SDS-sample buffer containing 5% (v/v) 2-mercaptoethanol and applied to SDS-PAGE.

Western Blotting

SDS-PAGE and Western blotting were performed as previously described [Saga et al., 1987].

Northern Blotting

Total RNA of cultured cells was extracted by the AGPC method. A human PDI [Miyaishi et al., 1995] and rat ERp61 [Kozaki et al., 1994] c-DNA probe has been described elsewhere. A cDNA of mouse ERp72 (1003bp) was synthesized from total RNA of mouse fibroblast by reverse transcription using Superscript^{TMII} (GIBCO BRL, Gaithersburg, MD) and amplified by PCR technique. Specific mouse ERp72 primers were used: 7201, 5′-TATGCACCATGGTGTGGACA-3′; 7202, 5′-AGGATGGCTGCATTGACATC-3′; 7203, 5′-CCATTGCTGTAGCGAAGATC-3′, and 7204, 5′-TACTCAGGGAAGTCCTTGGC-3′. The amplification condition consisted of 30 cycles of the following steps 94°C, 1 min; 54°C, 2 min; 72°C, 3 min. A set of primers, 7201 and 7202, was used for the first step of amplification and another set of primers, 7203 and 7204, for the second amplification. Total RNAs extracted by the AGPC method from F9 and dF9 cell cultures were electrophoresed in formaldehyde-agarose gels (1% w/v) and transferred to nylon membranes (Hybond-N$^+$, Amersham, Amersham, UK). The membranes were hybridized at 42°C overnight with ^{32}P-labelled probes under condition of 50% (v/v) formamide, 5 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA buffer, pH 7.4), 5×Denhalt's solution, 10% (v/v) dextran sulfate, 200 µg denatured salmon sperm DNA, and 0.1% (w/v) SDS. The membranes were washed with 0.1 × SSPE and 0.1% (w/v) SDS at 65°C.

RESULTS

F9 cells grew in culture forming tightly-packed colonies with homologous population however, undifferentiated F9 cells (F9 cells) treated with retinoic acid and dibutyryl cAMP for 4 days grew apart from one another and showed morphological alteration to polygonal flat cells with several processes. F9 cells and differentiated F9 cells (dF9 cells) were stained immunocytochemocally using affinity-purified rabbit antibodies against PDI, ERp61, and ERp72 as first antibodies, and followed by FITC-labeled affinity-purified goat antibody against rabbit IgG. PDI, ERp61, and ERp72 in undifferentiated F9 cells were similarly detected as weak staining of loose reticular networks surrounding the nucleus (Fig. 1A,C,E). In contrast, the immunostaining of dF9 cells by three antibodies was markedly enhanced and brightly stained reticular networks spread throughout the cytoplasm except for a part of the perinuclear area corresponding to Golgi apparatus, indicating their location in the endoplasmic

reticulum (ER; Fig 1B,D,F). These findings clearly demonstrated the distinct induction of PDI, ERp61, and ERp72 during differentiation.

To assess the induction of PDI, ERp61, and ERp72 proteins precisely, we radiolabeled the F9 and dF9 cells and analyzed by immunoprecipitation method. Aliquots of cell lysates with equal radioactivity of F9 and dF9 cells were used for immunoprecipitation for PDI, ERp61, and ERp72 (Fig. 2). The supernatant after immunoprecipitation contained no more remaining detectable amounts of antigen upon reprecipitation with the same antibodies (data not shown), ensuring the accuracy of the quantitative analysis of each antigen. PDI was detected as a single band with a molecular weight of 58 kDa, and strikingly increased during differentiation (lanes c and d). In dF9 cells, some other proteins coprecipitated with PDI were detected as weak bands. They were composed of several bands with a molecular weight of 60 to 65 kDa and a higher molecular weight of about 200 kDa (lane d). ERp61 was also expressed in constitutive amounts as well as PDI in F9 cells and similarly, or to a slightly lesser extent, increased during differentiation (lanes e and f).

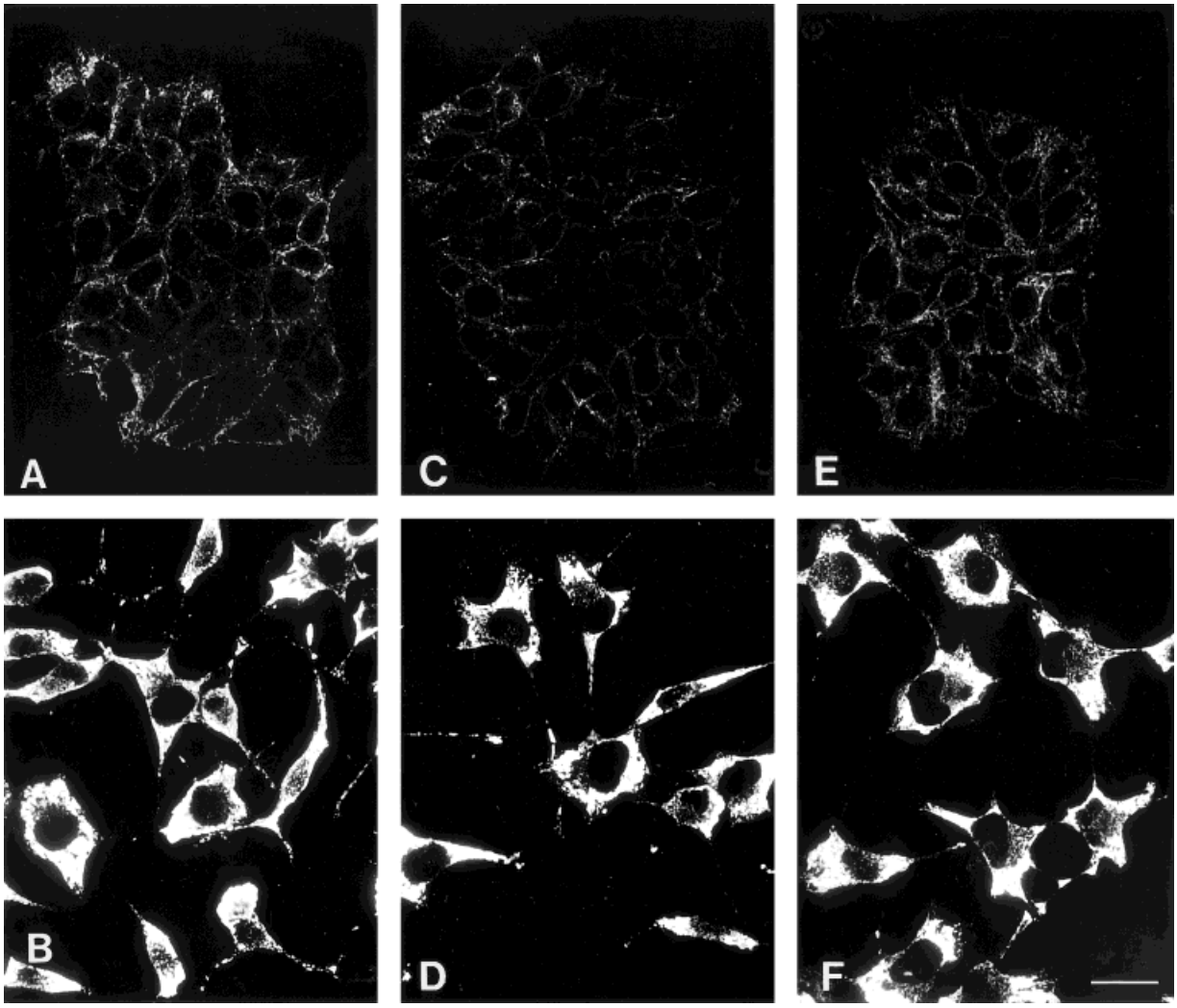

Fig. 1. Immunofluorescent micrograph of mouse F9 cells. The increase of PDI, ERp61, and ERp72 protein expression during differentiation of mouse F9 cells were investigated. F9 cells differentiated by the treatment with 2.5 μM retinoic acid and 2.5 mM dibutyryl cAMP for 4 days. The expressions of PDI, ERp61, and ERp72 were immunocytochemically examined. F9 **(A, C, E)** and differentiated F9 cells (dF9 cells) (B, D, F) cultured on coverslips and fixed with phosphate buffered formalin (12.5%) were immunostained with affinity-purified rabbit antibodies against PDI (A, **B**), ERp61 (C, **D**), and ERp72 (E, **F**). PDI, ERp61 and ERp72 were weakly stained in F9 cells, however, the immunoreactivities were markedly elevated in dF9 cells indicating increased expressions of the proteins during differentiation.

The co-precipitates with a molecular weight of about 200 kDa were faintly detected in the differentiated cells. ERp72 was also increased during differentiation (lanes g and h). A band with a molecular weight of 210 kDa was coprecipitated with ERp72, although much more in undifferentiated cells than in differentiated cells. The induction rates determined by densitometric analysis of PDI, ERp61, and ERp72 were 5.1, 3.0, and 2.7, respectively. We further investigated the mRNA levels of PDI, ERp61, and ERp72 during differentiation of F9 cells by Northern blot analysis, in which mRNAs of PDI, ERp61, and ERp72 were detected as single bands of 2.4, 2.6, and 2.8 kbp, respectively. As shown in Figure 3, the expression of mRNAs of PDI and ERp61 was clearly induced. On the other hand, ERp72 mRNA was not much increased during differentiation in contrast to the clear induction of ERp72 protein. We confirmed the slight increase of ERp72 mRNA by repeating the experiment three times. These results indicated that PDI, ERp61 and ERp72 were induced in different degrees in terms of the protein and mRNA levels during differentiation.

Since some proteins were co-immunoprecipitated with PDI family proteins as shown in Figure 2, dF9 cells were treated with chemical crosslinkers, DSP, DSS, and DTSSP followed by immunoprecipitation. If PDI, ERp61, or ERp72 interact intracellularly with other proteins, such proteins are expected to be more effectively coprecipitated with the three proteins by chemical crosslinking. Without crosslinking, very small amount of proteins were coprecipitated with PDI in dF9 cells (Fig. 2, lane d; Fig. 4, lane b). When the cells were treated with the crosslinking reagent DSP, membrane permeable and cleavable in an internal disulfide bond, distinct bands with a molecular weight of about 200 kDa were coprecipitated with PDI (Fig. 4, lane c). By crosslinking with DSS, a membrane permeable but not cleavable crosslinking reagent, PDI of authentic molecular size decreased and a broad band was detected at the boundary region between the stacking and resolving gels, suggesting the formation of a high molecular weight complex of proteins (Fig. 4, lane d). The bands coprecipitated with PDI in the cells treated with DTSSP, membrane impermeable and cleavable, were almost the same as those without crosslinking (Fig. 4, lane e). High molecular weight proteins of about 200 kDa were also co-immunoprecipitated with ERp61

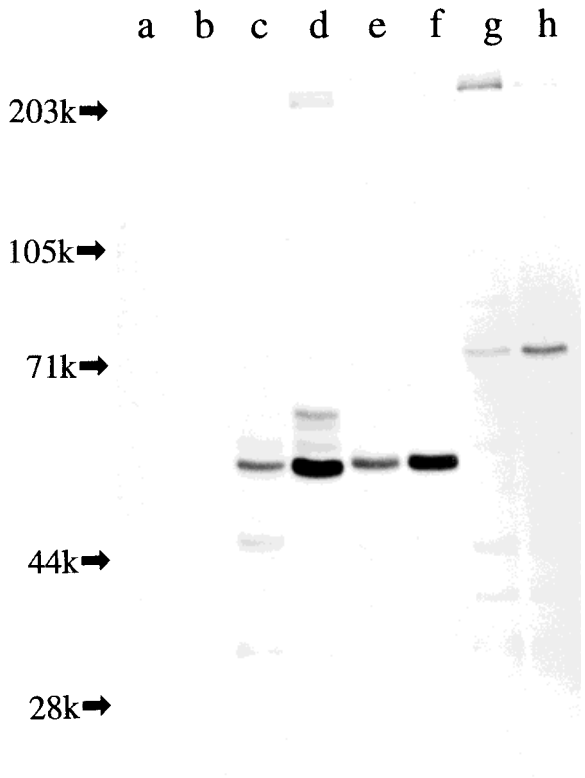

Fig. 2. The increased expression of PDI, ERp61, and ERp72 proteins in mouse F9 cells during differentiation. F9 and dF9 cells were radiolabeled for 8 h with 7.4 MBq/ml of Tran-^{35}S label at 37°C. To examine the induction of each protein, equal count of F9 cell lysates **(lane a, c, e, g)** and dF9 cell lysates **lane b, d, f, h)** were served for immunoprecipitation and analyzed by SDS-PAGE. In lane a and b; normal rabbit IgG was used for the control. Lane c and d, f fraction bound to anti-PDI antibody; lane e and f, fraction bound to anti-ERp61 antibody; lane g and h, fraction bound to anti-ERp72 antibody. The expression of PDI, ERp61, and ERp72 in F9 cells differs considerably. PDI and ERp61 were equally expressed, and the expression of ERp72 was weaker. However, the expression of all the proteins was elevated in dF9 cells as described in the text.

(Fig. 4, lane f) and ERp72 (Fig. 4, lane g) in dF9 cells with DSP treatment.

Since it has been reported that dF9 cells secrete much laminin and type IV collagen, and their molecular sizes are similar to those of the high molecular weight proteins co-precipitated with PDI family proteins, we compared the high molecular weight proteins with laminin and type IV collagen. Figure 5 demonstrates the high molecular weight proteins co-precipitated with PDI, ERp61, and ERp72 in dF9 cells, which were analyzed by SDS-PAGE using 5% resolving gel. To identify type IV collagen, immunoprecipitates were treated with bacterial collagenase. Co-precipitates with PDI were composed of several bands (Fig. 5, lane b), and two

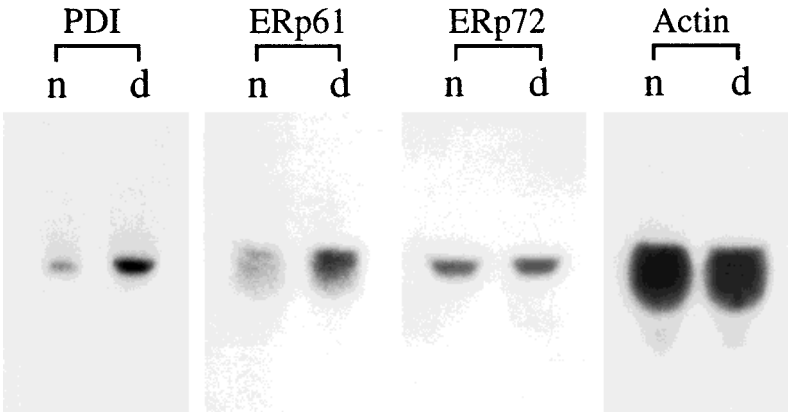

Fig. 3. The increased expression of PDI, ERp61, and ERp72 m-RNA in mouse F9 cells during differentiation. Equal amount (20 μg) of total RNAs extracted from F9 (n) and dF9 (d) cells were electrophoresed on a formaldehyde-agarose gels and blotted onto nylon membrane. The membrane was then hybridized to probes specific for PDI, ERp61, and ERp72. Exposure time were different for each probe. PDI, ERp61, and ERp72 showed induced expression in different degree. The expression of actin is shown as an internal control.

bands were digested by collagenase (Fig. 5, lane c). The molecular sizes of both bands were coincident with that of immunoprecipitates with anti-type IV collagen antibody (Fig. 5, lane k). Behind the larger band corresponding to α1(IV) chain that is sensitive to collagenase digestion, there were two other bands. These two bands were thought to be the B1 and B2 chains of laminin by comparing the immunoprecipitates with anti-laminin polyclonal antibody (Fig. 5, lanes h and i). In addition, the co-precipitates with PDI contained a higher molecular weight (about 400 kDa) corresponding to the A chain of laminin. We therefore concluded the co-immunoprecipitates of PDI were three polypeptides (A, B1, and B2) of laminin and two polypeptides (α1 and α2) of type IV collagen (Fig 5, lane A). The co-immunoprecipitates of ERp61 were similar to those of PDI with collagenase treatment (Fig. 5, lanes d, e, and B), indicating they were mainly composed of laminin polypeptides. The co-immunoprecipitates with ERp72 were also similar to those with PDI (Fig. 5, lanes f and g), however, further investigation revealed that they consisted of laminin, small amounts of type IV collagen and certain unidentified protein (Fig. 5, lane C and Fig. 2, lanes g and h). This band, with a molecular weight of about 210 kDa, was identical to the protein coprecipitated with ERp72 without crosslinker (Fig. 5, lane D), and was found to be resistant to collagenase (data not shown).

In order to confirm the identification of each co-precipitated polypeptide, the immunoprecipitates of DSP-crosslinked dF9 cells with anti-PDI, ERp61 and ERp72 antibodies were analyzed by Western blotting using antibody to laminin (Fig. 6, panel A) and antibody to type IV collagen (Fig. 6, panel B). In this system, laminin was detected as only a single band probably due to the loss of immunoreactivity in two of three polypeptides during the blotting procedure. This band was detected in all immunoprecipitates using the three antibodies. Although type IV collagen was also detected as duplicate bands in all immunoprecipitates, there was much more type IV collagen in the immunoprecipitate of PDI than in those of ERp61 or ERp72 proteins.

DISCUSSION

PDI, ERp61, and ERp72 form a protein family with amino acid sequence characterized by: 1) the presence of thioredoxin-like domains containing CGHC motifs as their active sites, and 2) the presence of an ER-retention signal in COOH-termini [Edman et al., 1985; Bennett et al., 1988; Mazzarella et al., 1990]. These characteristics imply that they function as the catalyst of the formation and isomerization of disulfide bonds in the ER. However, the specific functions of each protein in this family have not been elucidated. In the present study, we examined the alternative expression of PDI family proteins during differentiation of mouse F9 teratocarcinoma cells. F9 cells produce several proteins such as laminin, type IV collagen or plasminogen activator by the differentiation induced by retinoic acid and dibutyryl cAMP [Strickland et al., 1980; Wang et al., 1985]. If the PDI

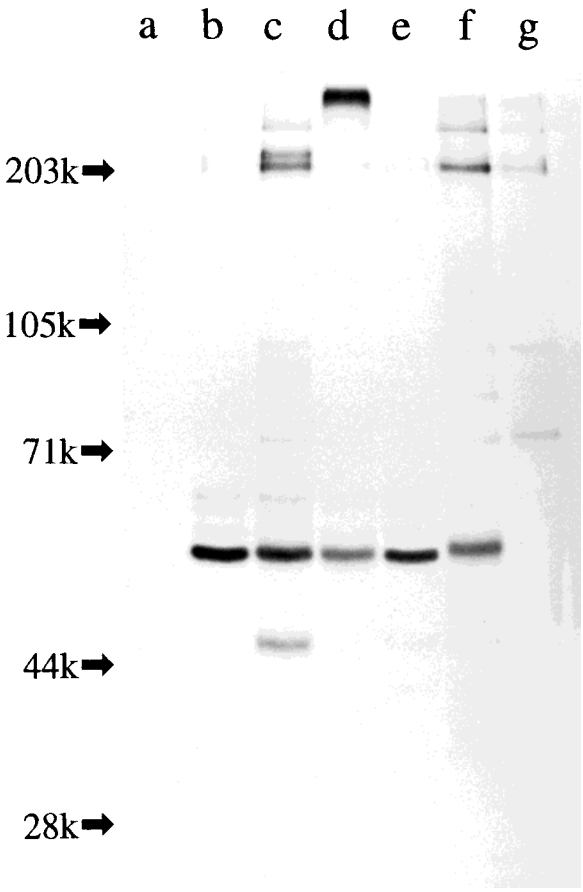

Fig. 4. Immunoprecipitation of dF9 cells pretreated with crosslinking reagents. Radiolabeled dF9 cells were pretreated with various crosslinkers, DSP, DSS, and DTSSP, for 30 min at 0°C and lysed in lysis buffer as described in Materials and Methods. For control experiments, the lysate of dF9 cells pretreated with DSP were immunoprecipitated with nonimmune rabbit IgG **(lane a)** and the lysate of dF9 cells without any pretreatment was immunoprecipitated with rabbit anti-PDI antibody **(lane b).** The lysate of dF9 cells pretreated with DSP were immunoprecipited with rabbit anti-PDI antibody **(lane c),** rabbit anti-ERp61 antibody **(lane f),** or rabbit anti-ERp72 antibody **(lane g).** The lysate of dF9 cells pretreated with DSS were immunoprecipited with anti-PDI antibody **(lane d)** and the lysate of dF9 cells pretreated with DTSSP were immunoprecipited with anti-PDI antibody **(lane e).**

family proteins interact with these proteins induced, the expression of PDI family proteins will also increase and if each member of the protein family possesses a specific role to some protein induced, the expression manner of each would be presumably different.

In this study, we first demonstrated the increase of PDI, ERp61, and ERp72 during the differentiation of mouse F9 teratocarcinoma cells. The elevated expression of PDI, ERp61, and ERp72 proteins in differentiated F9 (dF9) cells was demonstrated first by immunofluorescence and then more clearly by an immunoprecipitation method. In terms of protein level, the increase of the three protein expressions varied. PDI was most remarkably increased, then ERp61. ERp72 was significantly increased, but to a lesser extent. In mRNA levels, the expression of PDI, ERp61, and ERp72 differed considerably. The increase of PDI mRNA expression was the most remarkable and ERp61 mRNA was clearly induced, whereas the expression of ERp72 mRNA did not change. These results suggest that the expressions of PDI, ERp61, and ERp72 might be regulated in different ways.

We also demonstrated the co-precipitation of laminin and/or type IV collagen, both of which are intensively produced in dF9 cells, with PDI family proteins. Crosslinking and Western blotting experiments clearly demonstrated that laminin was equally coprecipitated with PDI, ERp61, and ERp72 whereas type IV collagen was coprecipitated with PDI and faintly with ERp61 and ERp72. These results imply that the three enzymes are generally concerned in laminin production, but PDI has a specific role on type IV collagen processing.

PDI family proteins have been suggested to possess biological functions other than thiol: protein disulfide oxidoreductase. PDI has been identified as a thyroid hormone-binding protein [Obata et al., 1988], a component of the microsomal triglyceride-transfer protein complex [Wetterau et al., 1990], and a glycosylation site-binding protein (GSBP) [Noiva et al., 1991] as well as the β-subunit of prolyl 4-hydroxylase [Pihlajaniemi et al., 1987]. In the case of ERp61 and ERp72, cysteine protease and carnitine palmitoyltransferase activity has been identified and found to be multifunctional [Urade et al., 1992, 1993; Murthy and Pande, 1994]. Furthermore, PDI family proteins are considered to possess a chaperone function. PDI was found to associate with misfolded lysozyme [Otsu et al., 1994] and to assist the refolding of denatured lysozyme in vitro [Puig and Gilbert, 1994]. ERp61 and ERp72 are induced by glucose starvation and treatment of tunicamycin or A23187 [Dorner et al., 1990; Van et al., 1993; Mazzarella et al., 1994]. Furthermore, in yeast, PDI, known to be indispensable for the viability of the organisms, is replaceable with mammalian PDI or ERp72 but not ERp61 [Gunther et al., 1993]. Therefore the association of PDI family proteins with other protein in this study might

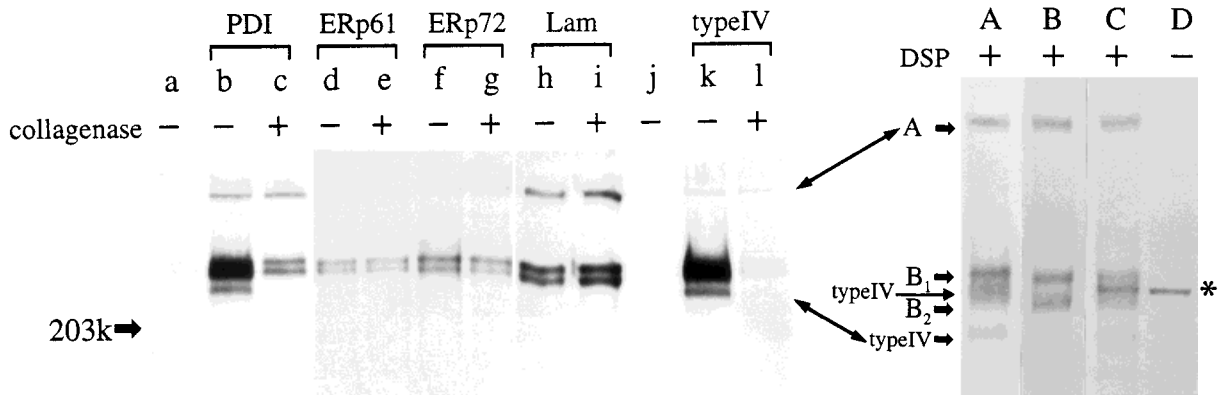

Fig. 5. The analysis of high molecular weight protein(s) immunoprecipitated from dF9 cell lysate pretreated with DSP. Radiolabeled dF9 cells were pretreated with DSP for 30 min at 0°C and lysed in lysis buffer as described in Materials and Methods. The lysate was immunoprecipitated using anti-PDI **(lanes b and c),** ERp61 **(lanes d and e),** or ERp72 **(lanes f and g)** antibodies. For control experiments, dF9 cell lysate pretreated with DSP was immunoprecipitated with nonimmune rabbit IgG **(lane a),** the lysate of dF9 cells without any pretreatment was immunoprecipitated with rabbit anti-laminin antibody **(lanes h and i),** with nonimmune goat IgG **(lane j),** and with goat anti-type IV collagen antibody **(lanes k and l).** The immunoprecipitates were digested with bacterial collagenase as indicated in the upper column. For further analysis of the high molecular weight proteins, SDS-PAGE of 4% resolving gel was performed. **Lanes A, B, and C** represent the crosslinked immunoprecipitates using anti-PDI, ERp61, and ERp72, respectively. Three polypeptides of laminin were equally co-immunoprecipitated with the three proteins, however, two polypeptides of type IV collagen were clearly detected in lane A and faintly in lane C. The clear band between B1 and B2 chains of laminin in lane C was considered other than a type IV collagen polypeptide. A polypeptide of the same molecular weight was co-immunoprecipitated with anti-ERp72 antibody in the absence of DSP (**lane D,** asterisk).

represent each specific function of the PDI family proteins other than the redox activity.

However, in the processing of fibrillar procollagens, the formation of inter/intramolecular disulfide bonds in C-propeptides are necessary for the triple helix formation and an in vitro study indicated that PDI may form the disulfide bonds of the peptides [Koivu and Myllyla, 1987]. Type IV collagen molecules may also require the formation of inter/intramolecular disulfide bonds in C-terminal globular domains and N-terminal tail for the subsequent triple helix formation. PDI is the same molecule as the β subunit of prolyl 4-hydroxylase [Pihlajaniemi et al., 1987] and increases in the mRNA of α and β subunits of the enzyme during differentiation of F9 cells also have been reported [Helaakoski et al., 1990], hence the molecule coprecipitated with type IV collagen may be the β subunit of prolyl 4-hydroxylase. However, 10–100-fold more PDI is produced than the α subunit of prolyl 4-hydroxylase [Helaakoski et al., 1990]. It is therefore unlikely that the coprecipitation of type IV collagen might represent a binding to the heterotetramer of prolyl 4-hydroxylase. Although the enzyme that forms disulfide bonds of procollagen propeptides in vivo is unidentified, our results suggest that the disulfide bond formation is mainly catalyzed by PDI, not by ERp61 and ERp72. Laminin is composed of three different polypeptides, each of them involving several globular domains and helical domains that contain much cysteine residue [Sasaki et al., 1987a, 1988, Sasaki and Yamada, 1987b]. Appropriate bridging formation of disulfide bonds is necessary for the native conformation of each polypeptide and subsequent assembling of three polypeptides. Because similar amounts of laminin were associated with the three proteins, three enzymes may be equally involved in disulfide bond formation of the laminin polypeptides.

Previously an investigation of tissue distribution of PDI family proteins drew attention to the specific role of PDI family proteins [Kozaki et al., 1994; Iida et al., 1996]. PDI family proteins were ubiquitously and abundantly distributed in various cell types such as thyroid follicular epithelia cells, mucus-secreting cells, and some kinds of neuroendocrine cells. They are known to secrete thyroglobulins, core proteins of mucins and chromogranins that are rich in intra- and/or intermolecular disulfide bonds. However, in the pancreas, intestines and testis, the PDI family proteins distribute in different cell types. The facts that the ERp61 staining in A cells of pancreatic islets was weaker than in B cells, and that the staining for PDI in islet cells

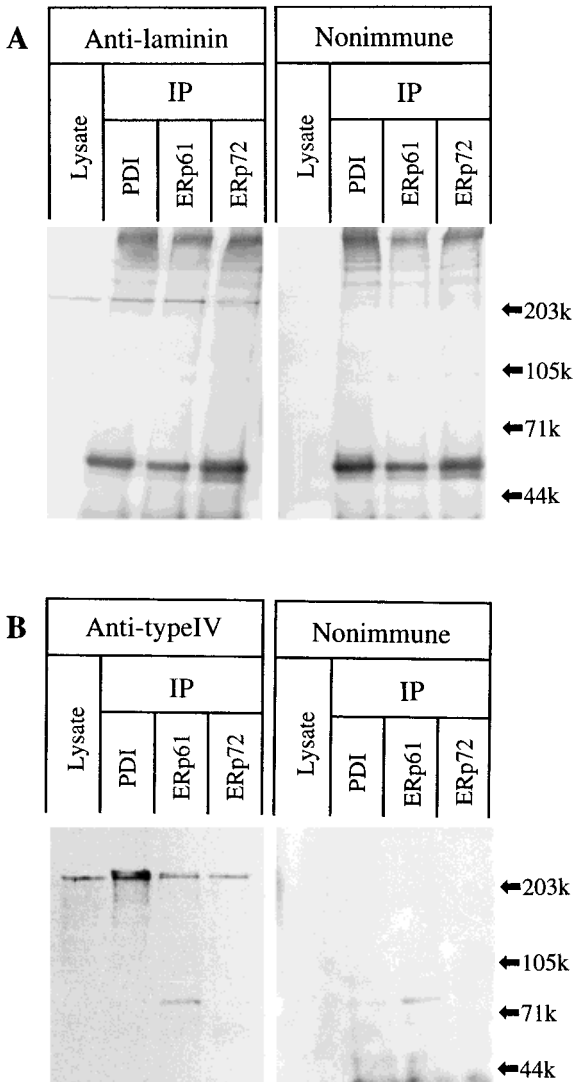

Fig. 6. Western blotting of immunoprecipitates. Immunoprecipitates using anti-PDI, ERp61, and ERp72 antibodies were subjected to SDS-PAGE and blotted to nitrocellulose membrane. The blotted membranes were then reacted with rabbit anti-laminin antibody (**A,** left) and with anti-type IV collagen antibody (**B,** left). The right halves of both panels are control that were reacted with nonimmune rabbit IgG and with nonimmune goat IgG, respectively. All the membranes were visualized by SABC method. Lysate, cell lysate of dF9 cells as a positive control; PDI, immunoprecipitates using anti-PDI antibody, ERp61, immunoprecipitates using anti-ERp61 antibody, ERp72, immunoprecipitates using anti-ERp72 antibody.

was very weak [Kozaki et al., 1994; Iida et al., 1996], leads to the hypothesis that PDI might not be essential for disulfide bond formation in the proteins produced in such cells, moreover, ERp61 could play an important role in producing insulin in B cells. Also, in IgA and IgM producing plasma cells, ERp61 and ERp72 were brightly stained, although PDI was stained weakly [Iida et al., 1996]. These immunohistochemical investigations also had suggested a substrate preference of each member of PDI family proteins. We therefore consider that the association of PDI family proteins with other secretory proteins shown in the present study may provide important information on their specific roles such as substrate specificity as thiol:protein disulfide oxidoreductase.

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