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Site-directed mutagenesis of human protein disulphide isomerase: effect on the assembly, activity and endoplasmic reticulum retention of human prolyl 4-hydroxylase in *Spodoptera frugiperda* insect cells

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Protein disulphide isomerase (PDI) is a highly unusual multifunctional polypeptide, identical to the β-subunit of prolyl 4-hydroxylase. It has two -Cys-Gly-His-Cys- sequences which represent two independently acting catalytic sites of PDI activity. We report here on the expression in baculovirus vectors of various mutant PDI/β-subunits together with a wild-type α-subunit of the human prolyl 4-hydroxylase $\alpha_2\beta_2$ tetramer in *Spodoptera frugiperda* insect cells. When either one or both of the -Cys-Gly-His-Cys- sequences was converted to -Ser-Gly-His-Cys-, a tetramer was formed as with wild-type PDI/β-subunit. This tetramer was fully active prolyl 4-hydroxylase. The data demonstrate that PDI activity of the PDI/β-subunit is not required for tetramer assembly or for the prolyl 4-hydroxylase activity of the tetramer, and thus other sequences of the PDI/β-subunit may be critical for keeping the α-subunits in a catalytically active, non-aggregated conformation. Measurements of the PDI activities of tetramers containing the various mutant PDI/β-subunits demonstrated that the activity of the wild-type tetramer is almost exclusively due to the C-terminal PDI catalytic sites, which explains the finding that the PDI activity of the PDI/β-subunit present in the tetramer is about half that in the free polypeptide. The data further demonstrate that one function of the PDI/β-subunit in prolyl 4-hydroxylase is to retain the enzyme within the endoplasmic reticulum, as deletion of the C-terminal -KDEL sequence of this polypeptide led to secretion of considerable amounts of both the free polypeptide and the active enzyme tetramer, whereas no secretion of either of these was found when the -KDEL sequence was present, and little or no secretion was found when the sequence had been modified to -HDEL.

Key words: baculovirus expression/collagen/glycosylation site-binding protein/microsomal triglyceride transfer protein/thyroid hormone-binding protein

Introduction

Protein disulphide isomerase (PDI, EC 5.3.4.1) is an abundant component of endoplasmic reticulum lumen, and is regarded as the *in vivo* catalyst for disulphide bond formation in the biosynthesis of various secretory and cell surface proteins. It catalyses thiol:disulphide interchange *in vitro*, leading to net protein disulphide bond formation, reduction or isomerization, depending on the reaction conditions (for reviews see Freedman, 1989; Freedman *et al.*, 1989; Noiva and Lennarz, 1992). Molecular cloning and nucleotide sequencing were first reported for rat PDI (Edman *et al.*, 1985). Subsequently PDI was found to be identical to the β-subunit of prolyl 4-hydroxylase (Koivu *et al.*, 1987; Pihlajaniemi *et al.*, 1987) and a cellular thyroid hormone-binding protein (Cheng *et al.*, 1987; Yamauchi *et al.*, 1987). PDI is the smaller subunit of the microsomal triglyceride transfer protein complex (Wetterau *et al.*, 1990, 1991) and is a polypeptide found in the lumen of the endoplasmic reticulum which uniquely binds various peptides (Geetha-Habib *et al.*, 1988; Noiva *et al.*, 1991).

The human PDI polypeptide consists of 491 amino acid residues and is synthesized in a form containing a signal peptide of 17 additional amino acids (Pihlajaniemi *et al.*, 1987). It has two thioredoxin-like regions (Edman *et al.*, 1985; Pihlajaniemi *et al.*, 1987; Parkkonen *et al.*, 1988), each containing a -Cys-Gly-His-Cys- sequence, and it has been proposed that these may act as catalytic sites for the isomerase activity (Freedman *et al.*, 1989; Hawkins and Freedman, 1991). Recently, oligonucleotide-directed mutagenesis was used to convert either one or both of the -Cys-Gly-His-Cys- sequences of human PDI expressed in *Escherichia coli* to -Ser-Gly-His-Cys-; the data demonstrated that both -Cys-Gly-His-Cys- sequences act as catalytic sites which appear to operate independently of one another (Vuori *et al.*, 1992a). The C-terminus of the PDI polypeptide has the sequence -KDEL, or in yeast, -HDEL (see Kivirikko *et al.*, 1990; Noiva and Lennarz, 1992) which acts as a retention signal for proteins of the endoplasmic reticulum lumen (Munro and Pelham, 1987; Pelham, 1989, 1990).

Prolyl 4-hydroxylase (EC 1.14.11.2), an $\alpha_2\beta_2$ tetramer, catalyses the formation of 4-hydroxyproline in collagen and related proteins by the hydroxylation of proline residues in peptide linkages. This modification plays a central role in collagen synthesis, as the 4-hydroxyproline residues formed are essential for the folding of the newly synthesized procollagen polypeptide chains into triple-helical molecules. The α-subunits contribute a major part to the two catalytic sites of the $\alpha_2\beta_2$ tetramer, but some parts of the large catalytic sites may be co-operatively built up of both α- and β-subunits (for reviews see Kivirikko *et al.*, 1989, 1992).

The role of the multifunctional PDI/β-subunit in the prolyl 4-hydroxylase tetramer is poorly understood. Some data suggest that PDI activity of the PDI/β-subunit may not be involved in the catalytic mechanism of the prolyl 4-hydroxylase reaction (Myllylä *et al.*, 1989), but investigation of this hypothesis has been limited by the inability of constructing an enzyme tetramer from its subunits *in vitro* (see Kivirikko *et al.*, 1992). This association problem was nevertheless solved very recently by expressing the two types of subunit of human prolyl 4-hydroxylase in *Spodoptera frugiperda* insect cells using baculovirus vectors (Vuori

et al., 1992b). We have now studied a possible role for the two PDI catalytic sites of the PDI/β-subunit in tetramer assembly and prolyl 4-hydroxylase catalytic activity by means of site-directed mutagenesis of these sequences in the baculovirus expression system. In addition, we have studied a possible role for the C-terminal -KDEL sequence of the PDI/β-subunit, which is not present in the α-subunit (Helaakoski *et al.*, 1989), in the retention of the prolyl 4-hydroxylase tetramer within the endoplasmic reticulum.

Results

Protein disulphide isomerase activity of the PDI/β-subunit is not required for tetramer assembly or prolyl 4-hydroxylase activity in the enzyme tetramer

In order to study the possible role of the two PDI catalytic sites of PDI/β-subunit in tetramer association and prolyl 4-hydroxylase catalytic activity, three recombinant viruses coding for various mutant human PDI/β polypeptides were generated and subjected to three rounds of plaque purification. In PDI/βN the sequence coding for the N-terminal catalytic site of PDI activity was modified by replacing the first Cys codon with a Ser codon; the PDI/βC virus had the same change made to the first Cys codon in the C-terminal catalytic site; and PDI/βNC had the changes made to both catalytic sites (Figure 1A). The three recombinant viruses encoding the mutant PDI/β-subunits were then used to infect *S.frugiperda* Sf9 insect cells, and the PDI activities of the resulting polypeptides were measured in the 0.1% Triton X-100-soluble protein fraction of the cell homogenates. The enzyme activities of the three PDI/β-subunit variants were found to be identical to those reported for the corresponding polypeptides expressed in *E.coli* (Vuori *et al.*, 1992a), i.e. the PDI activities of the PDI/βN and PDI/βC polypeptides were 50% of that of the wild-type polypeptide, whereas the PDI/βNC polypeptide showed no PDI activity (details not shown).

Each of the recombinant viruses encoding the wild-type polypeptide or a mutant PDI/β-subunit were then used to co-infect the Sf9 cells together with a recombinant virus encoding the α-subunit of human prolyl 4-hydroxylase, as reported previously (Vuori *et al.*, 1992b), and the 0.1% Triton X-100-soluble proteins of the cell homogenates were studied by PAGE on 7.5% acrylamide under non-denaturing conditions (Figure 1B). A distinct band corresponding to the enzyme tetramer was seen in the 0.1% Triton X-100-soluble protein fractions from cells infected with any of the PDI/β-subunit-encoding viruses, no differences were found in the intensity of this band between the four α-subunit and PDI/β-subunit combinations (Figure 1B). A band in Coomassie staining corresponding to the unassociated PDI/β-subunits was found just below a major polypeptide band, which was also produced by the wild-type virus (lane 5 in Figure 1B), the intensity of the PDI/β polypeptide band being slightly less than that of the tetramer band (Figure 1B). As reported previously (Vuori *et al.*, 1992b), the unassociated α-subunits form aggregates that are insoluble in 0.1% Triton X-100, and hence no band corresponding to them is present. Bands corresponding to the enzyme tetramer and unassociated PDI/β-subunits were also seen by Western blotting (lanes 7–10 in Figure 1B), but here the intensity of the band corresponding to the free PDI/β-subunit was much stronger than that of the tetramer band, due to a much stronger reaction of the monoclonal antibody 5B5 (Höyhtyä *et al.*, 1984) with the free PDI/β-subunit than with the enzyme tetramer.

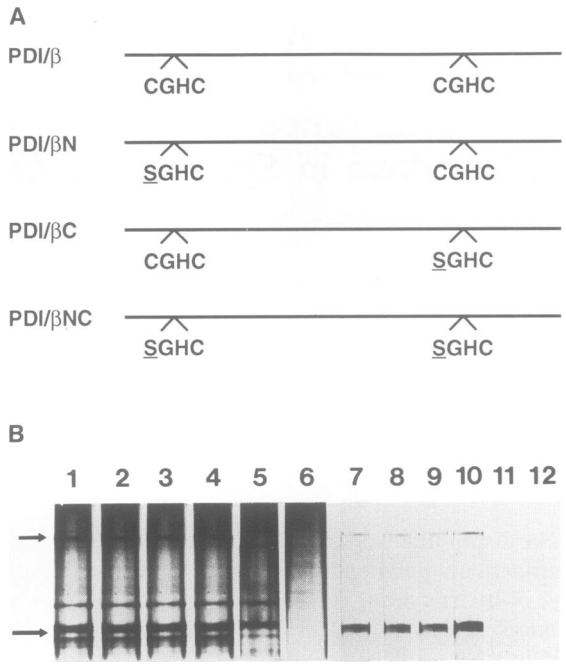

Fig. 1. PAGE analysis under non-denaturing conditions of tetramer formation from wild-type α-subunits and wild-type or mutant PDI/β-subunits of human prolyl 4-hydroxylase expressed in Sf9 cells by means of baculovirus vectors. In (**A**) the catalytic sites of the various mutant PDI/β-subunits are shown with the altered amino acids underlined. In (**B**) lanes 1–6 show Coomassie staining and lanes 7–12 Western blotting of PAGE run on 7.5% acrylamide. Lanes 1–4 and 7–10 show 0.1% Triton X-100-soluble proteins from cells infected with PDI/β, PDI/βN, PDI/βC or PDI/βNC virus together with an α-subunit-encoding virus respectively. Lanes 5 and 11 show the corresponding fraction from cells infected with the wild-type AcNPV virus, and lanes 6 and 12 from mock-infected cells. The cells were homogenized 72 h after infection. The monoclonal antibody 5B5 used in Western blotting reacts much more strongly with the free PDI/β-subunit than with the enzyme tetramer. The arrows indicate the positions of the enzyme tetramer and the free PDI/β-subunit.

The four types of prolyl 4-hydroxylase tetramer were then purified to homogeneity (Figure 2) by a previously reported affinity chromatography procedure (see Kivirikko and Myllylä, 1982, 1987), and the purified enzyme tetramers were assayed for prolyl 4-hydroxylase activity. The maximal velocities of all four types of the enzyme tetramer were found to be identical (Table I), and their K_m values for Fe^{2+}, 2-oxoglutarate, ascorbate and the peptide substrate were also identical (details not shown).

PDI activities of the native enzyme tetramers containing the various mutant PDI/β-subunits

It was previously found that the PDI activity of the PDI/β-subunit present in the native human prolyl 4-hydroxylase tetramer is only about half that of the free human PDI/β-subunit (Koivu *et al.*, 1987). This raised the possibility that one of the two catalytic sites of the PDI/β-subunit might be unavailable for reaction when the polypeptide is present in the prolyl 4-hydroxylase tetramer, while the other catalytic site was fully active. To test this hypothesis, the enzyme tetramers containing the various mutant PDI/β-subunits were assayed for PDI activity. In agreement with the data reported

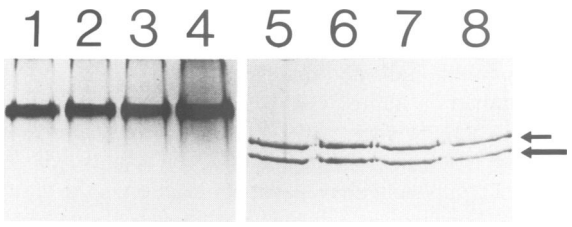

Fig. 2. PAGE analysis under non-denaturing conditions and SDS−PAGE analysis of affinity column purified recombinant prolyl 4-hydroxylase tetramers. The samples in lanes 1−4 were run on 7.5% PAGE and those in lanes 5−8 on 10% SDS−PAGE, and both gels were analysed by Coomassie staining. Lanes 1 and 5, 2 and 6, 3 and 7, and 4 and 8 contain purified prolyl 4-hydroxylase from Sf9 cells infected with PDI/β, PDI/βN, PDI/βC or PDI/βNC virus together with an α-subunit-encoding virus respectively. The locations of the α- and β-subunits on the SDS−PAGE are shown on the right by the short and long arrows respectively.

Table I. Prolyl 4-hydroxylase and PDI activities of enzyme tetramers containing various mutant PDI/β-subunits

Polypeptide or tetramer[a]	Prolyl 4-hydroxylase activity[b] (mol/mol/s)	Relative PDI activity[c] (%)
Free PDI/β polypeptide	0	100
α_2(PDI/β)$_2$	12.8 ± 0.1	53 ± 3
α_2(PDI/βN)$_2$	12.7 ± 0.1	50 ± 2
α_2(PDI/βC)$_2$	12.8 ± 0.1	8 ± 1
α_2(PDI/βNC)$_2$	12.7 ± 0.2	0

[a]Enzyme tetramers as described in the text.
[b]V_{max}, mean of two experiments ± the limit of range.
[c]Taking pure PDI/β polypeptide as 100%. Mean of two experiments, ± the limit of range.

previously for the proteins when purified from human placental tissue (Koivu *et al.*, 1987), the PDI activity of the PDI/β-subunit present in the recombinant enzyme tetramer was found to be ~50% of that of the free PDI/β polypeptide (Table I). The PDI activity of the enzyme tetramer containing two PDI/β-subunits with the mutant N-terminal catalytic site was likewise ~50% of that of the free wild-type polypeptide, whereas the activity of the tetramer with two PDI/βC-subunits was only 5−10% (Table I). The latter activity was still higher than that of the tetramer with two PDI/βNC-subunits, however, which showed no PDI activity at all (Table I).

The C-terminal -KDEL sequence of the PDI/β-subunit is required for the cellular retention of the prolyl 4-hydroxylase tetramer

In order to study the possible role of the C-terminal -KDEL sequence of the PDI/β-subunit in the retention of the prolyl 4-hydroxylase tetramer within the endoplasmic reticulum, two recombinant viruses encoding mutant PDI/β polypeptides were generated and subjected to three rounds of plaque purification. The PDI/βHDEL polypeptide had its C-terminal -KDEL sequence altered to -HDEL, while PDI/βΔKDEL had the last four amino acids of the polypeptide deleted. Each of these viruses were then infected into Sf9 cells together with an α-subunit-encoding virus, and the 0.1% Triton X-100 extracts of cell homogenates and the concentrated medium samples were studied 24−96 h after infection by Western blotting of PAGE performed on 7.5% acrylamide

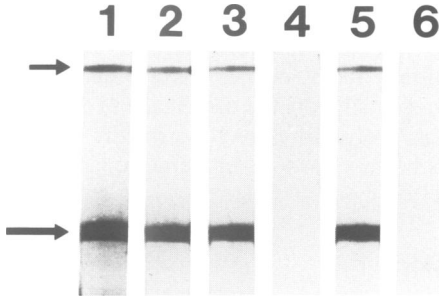

Fig. 3. Western blot analysis of recombinant prolyl 4-hydroxylase tetramers with varying C-terminal sequences in the PDI/β-subunit. The samples were run on 7.5% acrylamide PAGE under non-denaturing conditions and analysed by Western blotting with the monoclonal antibody 5B5 to the human PDI/β-subunit. Lanes 1−3 show 5 μl out of 500 μl of 0.1% Triton X-100-soluble proteins from Sf9 cells infected with PDI/β, PDI/βΔKDEL or PDI/βHDEL virus together with an α-subunit-encoding virus respectively; and lanes 4−6 show 10 μl of the corresponding medium samples concentrated to one-eighth of their original 2 ml volume. All the samples were analysed 72 h after infection. The arrows indicate the positions of the enzyme tetramer and the free PDI/β-subunit.

gels under non-denaturing conditions. In agreement with previous data (Vuori *et al.*, 1992b), no secretion of either the free PDI/β polypeptide or the enzyme tetramer into the culture medium was found at any of the time points between 24 and 72 h when the -KDEL sequence was present in the PDI/β-subunit (as shown for 72 h after infection in Figure 3). However, trace amounts of the free PDI/β-subunit were seen at 96 h in some but not all of the highly concentrated medium samples (20-fold concentration, details not shown), since some of the cells appeared to be dying, it is possible that this was due to cell lysis rather than secretion. Similarly, secretion of either the free PDI/β-subunit or the enzyme tetramer was not usually detected until 72 h when the -KDEL sequence had been modified to -HDEL (Figure 3), but trace amounts of the free PDI/β polypeptide were seen in highly concentrated (20-fold) medium samples at 72 and 96 h in some experiments (details not shown). By contrast, when the -KDEL sequence had been deleted, distinct secretion of both the free PDI/β polypeptide and the enzyme tetramer was detectable ~48 h after infection (as shown for 72 h in Figure 3). It should be noted that the ratios of the intensities of the bands corresponding to the free polypeptide and the enzyme tetramer in Figure 3 are highly misleading, as the monoclonal antibody 5B5 reacts much more readily with the free PDI/β-subunit than with the enzyme tetramer (see above and Figure 1). The intensity ratios of these two bands in the medium samples were found to be essentially the same as in the cell homogenates, suggesting that the actual amounts of free polypeptide and enzyme tetramer in the medium were roughly equal. The specific PDI activities of the polypeptides with either the C-terminal -HDEL sequence or deletion of these four amino acids were identical to that of the wild-type PDI/β polypeptide, and the prolyl 4-hydroxylase activities of the enzyme tetramers containing the PDI/β-subunits with the C-terminal -HDEL sequence or deletion of these four amino acids were likewise identical to that of the wild-type enzyme tetramer (details not shown). In the case of infection with the PDI/βΔKDEL virus together with the α-virus, a significant amount of prolyl 4-hydroxylase activity was also found in

the culture medium. About 90% of the total enzyme activity was found in the cell fraction 72 h after infection and 10% in the culture medium, but these values are likely to underestimate markedly the percentage of the enzyme in the medium, as prolyl 4-hydroxylase rapidly loses its activity in various buffers at room temperature (see Kivirikko and Myllylä, 1982).

Discussion

The PDI/β-subunit is now known to be a highly unusual multifunctional polypeptide (see Introduction), but apart from its role as the enzyme PDI, details of its other functions are poorly understood. In the case of prolyl 4-hydroxylase, it was recently found that α-subunits expressed via the baculovirus system in insect cells without the PDI/β-subunits form highly insoluble, catalytically inactive aggregates which can only be solubilized with 1% SDS (Vuori *et al.*, 1992b). This behaviour differs distinctly from that of the free PDI/β polypeptide or the prolyl 4-hydroxylase tetramer, which are both highly soluble in all cell types studied (see Kivirikko and Myllylä, 1982; Kivirikko *et al.*, 1992), including insect cells (Vuori *et al.*, 1992b), provided that the membranes of the endoplasmic reticulum have been disrupted. The PDI/β-subunits thus appear to be required in the prolyl 4-hydroxylase tetramer to keep the α-subunits in a catalytically active, non-aggregated conformation (Vuori *et al.*, 1992b). No data have been available to indicate whether one or both of the PDI catalytic sites of the PDI/β-subunit participate in this function.

The present data clearly demonstrate that PDI activity in the PDI/β-subunit is not required for association of the α- and PDI/β-subunits into the enzyme tetramer, neither is this activity needed for prolyl 4-hydroxylase activity in the enzyme tetramer. It thus appears that other sequences of the PDI/β-subunit may be critical for tetramer assembly and for keeping the α-subunits in a catalytically active, non-aggregated conformation. In agreement with this, preliminary data from Lennarz's laboratory (see Noiva and Lennarz, 1992; Noiva *et al.*, 1991) indicate that the unspecific peptide-binding property of the PDI/β polypeptide, which may be related to the function of this subunit in prolyl 4-hydroxylase, may not involve the PDI catalytic sites. These conclusions are also likely to hold true for the similar function of the PDI/β-subunit in the microsomal triglyceride transfer protein complex dimer (Wetterau *et al.*, 1991), which appears to be very similar to the role of this subunit in the prolyl 4-hydroxylase tetramer, since recent *in vitro* experiments demonstrate that when the triglyceride transfer protein complex is dissociated the 88 kDa subunit immediately forms catalytically inactive, insoluble aggregates (Wetterau *et al.*, 1991).

Although the data demonstrate that PDI activity of the human PDI/β-subunit of prolyl 4-hydroxylase is not required for tetramer assembly, the results do not completely exclude a role for PDI in the assembly process. Sf9 cells have a low level of endogenous PDI activity (details not shown) which may play some role in assembly. Nevertheless, our previous work demonstrated that this endogenous PDI activity is not able to maintain a detectable level of the α-subunit of human prolyl 4-hydroxylase in a catalytically active, non-aggregated conformation when the α-subunits are expressed in the insect cells without the human PDI/β-subunit (Vuori *et al.*, 1992b).

Measurements of the PDI activities of the prolyl 4-hydroxylase tetramers containing the various mutant PDI/β-subunits indicated that the PDI activity of the wild-type tetramer is almost exclusively attributable to the C-terminal catalytic sites of the PDI/β-subunits, as inactivation of the N-terminal site had no effect on activity. The latter site is not completely unavailable for reaction in the tetramer structure, however, as a distinct activity was observed when the C-terminal site had been inactivated. The microsomal triglyceride transfer protein dimer differs from the prolyl 4-hydroxylase tetramer in this respect, as it shows no PDI activity in the native state, although full activity is recovered after dissociation of the dimer (Wetterau *et al.*, 1991).

Proteins residing within the endoplasmic reticulum lumen are now known to be retrieved from the traffic of secretory proteins by interaction of their C-terminal -KDEL sequence or its variants with specific receptors (Munro and Pelham, 1987; Pelham, 1989, 1990). Membrane-bound proteins with characteristics of retention signal receptors have recently been identified from both mammalian cells (Vaux *et al.*, 1990) and yeast (Lewis *et al.*, 1990; Semenza *et al.*, 1990), and the -KDEL sequence of the PDI/β polypeptide has now been clearly shown to be critical for the retention of this protein within the endoplasmic reticulum (Mazzarella *et al.*, 1990; Haugejorden *et al.*, 1991). The α-subunit of prolyl 4-hydroxylase has no C-terminal -KDEL-like motif, the sequence being -SELE both in human (Helaakoski *et al.*, 1989) and chick subunits (Bassuk *et al.*, 1989). This has raised the possibility that the -KDEL sequence of the PDI/β-subunit may be required for retention not only of the free polypeptide but also of the prolyl 4-hydroxylase tetramer. The present data demonstrate that this hypothesis appears to be correct, and thus one function of the PDI/β-subunit in the prolyl 4-hydroxylase tetramer is to retain the enzyme tetramer within the lumen of the endoplasmic reticulum. No secretion of either the free PDI/β-subunit or the enzyme tetramer into the culture medium was found when the -KDEL sequence was present, whereas considerable amounts of both the free polypeptide and active enzyme tetramer were secreted when the -KDEL sequence had been deleted. Nevertheless, the rate of this secretion was very slow compared with rates observed in experiments with cultured vertebrate cells (Mazzarella *et al.*, 1990; Haugejorden *et al.*, 1991). This may be due to at least two reasons. One is that the Sf9 insect cells in baculovirus expression experiments often secrete even secretory proteins poorly as compared with many vertebrate cells (Sissom and Ellis, 1989; Vernet *et al.*, 1990). Another reason is that the PDI/β polypeptide appears to contain other sequences which delay its secretion from the endoplasmic reticulum, as demonstrated in experiments with COS cells in which the rates of secretion of the PDI/β and ERp72 polypeptides were compared after removal of their C-terminal retention signals (Mazzarella *et al.*, 1990).

Since it was not known initially whether the insect cells would prefer the -KDEL retention signal or the -HDEL sequence utilized by the yeast *Saccharomyces cerevisiae* (Pelham, 1989), we also studied whether a C-terminal -HDEL sequence would lead to retention of the PDI/β polypeptide and the prolyl 4-hydroxylase tetramer within Sf9 cells. The presence of this sequence clearly prevented secretion as compared with deletion of the tetrapeptide signal. However, the very small amount of secretion noted in concentrated medium samples 72 h after infection suggests

that although both -HDEL and -KDEL were recognized as retention signals by the Sf9 cells, the latter may be slightly more effective than the former.

Materials and methods

Construction of baculovirus transfer vectors and generation of recombinant viruses

The baculovirus transfer vector encoding the α-subunit of human prolyl 4-hydroxylase contained 61 bp of 5′ untranslated sequence, the whole coding region and 551 bp of 3′ untranslated sequence of α-subunit cDNA PA-58 cloned to the *PstI*−*Bam*HI site of the baculovirus transfer vector pVL1392 (Luckow and Summers, 1989), as described by Vuori *et al.* (1992b). The baculovirus transfer vector encoding the PDI/β-subunit contained a 1772 bp *Eco*RI−*Bam*HI fragment with 44 bp of 5′ untranslated sequences, the whole coding region and 201 bp of 3′ untranslated sequence of the PDI/β-subunit cDNA cloned to the *Eco*RI−BamHI site of the vector pVL1392 (Vuori *et al.*, 1992b). In the baculovirus transfer vectors used to generate the recombinant viruses PDI/βN, PDI/βC and PDI/βNC, the codon for the first Cys at either the N-terminal or C-terminal catalytic site or both in the PDI/β-subunit was altered to the codon for Ser by site-directed mutagenesis (Amersham) as described by Vuori *et al.* (1992a).

The sequence encoding the endoplasmic reticulum retention signal -KDEL in the PDI/β-subunit was either deleted or altered to -HDEL by means of the *in vitro* mutagenesis system (Amersham) using an M13 vector containing the full-length PDI/β-subunit cDNA (Pihlajaniemi *et al.*, 1987) as a template. The *Eco*RI−*Bam*HI fragments of these mutant PDI/β-subunit cDNA clones were then cloned into the *Eco*RI−*Bam*HI site of pVL1392.

Recombinant transfer vectors were co-transfected into *S.frugiperda* Sf9 cells with wild-type AcNPV viral DNA by calcium phosphate transfection, and the recombinant viruses were selected as described by Summers and Smith (1987).

Expression and analysis of recombinant proteins in Sf9 cells

Sf9 cells were cultured in TNM-FH medium (Sigma) supplemented with 10% fetal calf serum at 27°C and were infected with the purified recombinant viruses at a multiplicity of infection of five. The α and PDI/β viruses were used for infection in a 1:1 ratio when producing the prolyl 4-hydroxylase tetramer. The cells were harvested 24−96 h after infection and homogenized in 10 mM Tris pH 7.8, 0.1 M NaCl, 0.1 M glycine, 10 μM dithiothreitol and 0.1% Triton X-100 and centrifuged at 1000 *g* for 10 min. The resulting supernatants were analysed on 10% SDS−PAGE or 7.5% native PAGE and assayed for enzyme activities. The cell medium 24−96 h post-infection was concentrated to 5−25% of its original volume, analysed on 10% SDS−PAGE or 7.5% native PAGE and assayed for prolyl 4-hydroxylase activity. The cells in these experiments were grown in TNM-FH medium without fetal calf serum. Monoclonal antibody 5B5 to the PDI/β-subunit (Höyhtyä *et al.*, 1984) was used in Western blot analysis.

Protein purification and enzyme activity assays

Prolyl 4-hydroxylase was purified from Sf9 cells infected with the recombinant prolyl 4-hydroxylase subunit viruses by a procedure consisting of poly(L-proline) affinity chromatography, DEAE−cellulose chromatography and gel filtration (see Kivirikko and Myllylä, 1987).

PDI activity was assayed by a method involving measurement of the rate of regeneration of incorrectly disulphide-linked RNase to the native form (Ibbetson and Freedman, 1976), and prolyl 4-hydroxylase activity by a method based on the decarboxylation of 2-oxo[1-^{14}C]glutarate (see Kivirikko and Myllylä, 1982). The K_m values were determined by varying the concentration of one substrate in the presence of fixed concentrations of the second while the concentrations of the other substrates were held constant (Myllylä *et al.*, 1977).

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