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Characterization of the human prolyl 4-hydroxylase tetramer and its multifunctional protein disulfide-isomerase subunit synthesized in a baculovirus expression system

(collagen/thyroid hormone-binding protein/glycosylation site-binding protein/microsomal triacylglycerol transfer protein)

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ABSTRACT **Prolyl 4-hydroxylase (EC 1.14.11.2), an $\alpha_2\beta_2$ tetramer, catalyzes the posttranslational formation of 4-hydroxyproline in collagens. The enzyme can easily be dissociated into its subunits, but all attempts to associate a tetramer from the dissociated subunits *in vitro* have been unsuccessful. Molecular cloning of the catalytically important α subunit has identified two types of cDNA clone due to mutually exclusive alternative splicing. The β subunit is a highly unusual multifunctional polypeptide, being identical to the enzyme protein disulfide-isomerase (EC 5.3.4.1). We report here on expression of the α and β subunits of prolyl 4-hydroxylase and a fully active enzyme tetramer in *Spodoptera frugiperda* insect cells by baculovirus vectors. When the β subunit was expressed alone, the polypeptide produced was found in a 0.1% Triton X-100 extract of the cell homogenate and was a fully active protein disulfide-isomerase. When either form of the α subunit was expressed alone, only traces of the α subunit could be extracted from the cell homogenate with 0.1% Triton X-100, and 1% SDS was required to obtain efficient solubilization. These α subunits had no prolyl 4-hydroxylase activity. When the cells were coinfected with both α- and β-subunit-producing viruses, an enzyme tetramer was formed, but significant amounts of α and β subunits remained unassociated. The recombinant tetramer was indistinguishable from that isolated from vertebrate tissue in terms of its specific activity and kinetic constants for cosubstrates and the peptide substrate. The two alternatively spliced forms of the α subunit gave enzyme tetramers with identical catalytic properties. Baculovirus expression seems to be an excellent system for mass production of the enzyme tetramer and for detailed investigation of the mechanisms involved in the association of the monomers.**

Prolyl 4-hydroxylase (EC 1.14.11.2), an enzyme residing in the lumen of the endoplasmic reticulum, catalyzes the formation of 4-hydroxyproline in collagens and related proteins by the hydroxylation of proline residues in peptide linkages. This cotranslational and posttranslational modification plays a crucial 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 active prolyl 4-hydroxylase is an $\alpha_2\beta_2$ tetramer consisting of two types of inactive monomer with molecular weights of about 61,000 (α subunit) and 55,000 (β subunit). The α subunits contribute a major part to the two catalytic sites of the enzyme tetramer, but some parts of these large catalytic sites may be cooperatively built up of both types of subunit (for recent reviews, see refs. 1–3).

Molecular cloning of the α subunit of human prolyl 4-hydroxylase has identified two types of cDNA clone that differ in a stretch of 64 base pairs (bp) due to mutually exclusive alternative splicing (4). The two types of mRNA were present in human skin fibroblasts in approximately equal amounts, which suggested that the prolyl 4-hydroxylase $\alpha_2\beta_2$ tetramer may contain one α subunit of each type (2, 4). The β subunit is a highly unusual multifunctional polypeptide, being identical to the enzyme protein disulfide-isomerase (EC 5.3.4.1) (5–7), a cellular thyroid hormone-binding protein (8, 9), a component of the microsomal triacylglycerol transfer protein complex (10, 11), a dehydroascorbate reductase (12), and an endoplasmic reticulum luminal polypeptide uniquely binding various peptides (13, 14). Recent reviews are available on this multifunctional polypeptide (1–3, 15, 16).

The prolyl 4-hydroxylase tetramer can easily be dissociated into monomers by either reduction, alkylation, treatment with LiCl, or lowering of the pH (17–19). All attempts to construct an active enzyme tetramer from its subunits *in vitro* have been unsuccessful, however (3, 19, 20). The multifunctional β subunit has recently been expressed in *Escherichia coli* in a fully active form in terms of its protein disulfide-isomerase activity, and site-directed mutagenesis has been used to elucidate sequences involved in the catalytic sites for the isomerase activity (21). We report here on expression of the multifunctional β subunit and a fully active prolyl 4-hydroxylase tetramer in the insect cell line Sf9 (from *Spodoptera frugiperda*) by baculovirus vectors. Our data indicate that α subunits expressed without β subunits have no prolyl 4-hydroxylase activity and that either of the alternatively spliced forms of the α subunit will associate with the β subunits to yield a fully active enzyme tetramer.

MATERIALS AND METHODS

Construction of the Baculovirus Transfer Vectors pVLα58, pVLα59, and pVLβ. The baculovirus transfer vector pVLα58 was constructed by digesting a pBluescript (Stratagene) vector containing in the *Sma* I site the full-length cDNA for the α subunit of human prolyl 4-hydroxylase, PA-58 (4), with *Pst* I and *Bam*HI, the cleavage sites for which closely flank the *Sma* I site. The resulting *Pst* I–*Pst* I and *Pst* I–*Bam*HI fragments containing 61 bp of the 5′ untranslated sequence, the whole coding region, and 551 bp of the 3′ untranslated sequence were cloned to the *Pst* I–*Bam*HI site of the baculovirus transfer vector pVL1392 (22). The baculovirus transfer vector pVLα59 was similarly constructed from pVL1392 and another cDNA clone, PA-59 (4), encoding the α subunit of human prolyl 4-hydroxylase. The cDNA clones PA-58 and PA-59 differ by a stretch of 64 bp (see Introduction).

The pVLβ vector was constructed by ligation of an *Eco*RI–*Bam*HI fragment of a full-length cDNA for the β subunit of

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human prolyl 4-hydroxylase, S-138 (5), containing 44 bp of the 5′ untranslated sequence, the whole coding region, and 201 bp of the 3′ untranslated sequence to *Eco*RI/*Bam*HI-digested pVL1392.

Transfection and Isolation of Recombinant Viruses. Recombinant baculovirus transfer vectors were cotransfected into Sf9 cells (23) with wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV) DNA by calcium phosphate transfection. The resultant viral pool in the supernatant of the transfected cells was collected 4 days later and used for plaque assay. Recombinant occlusion-negative plaques were subjected to three rounds of plaque purification to generate recombinant viruses totally free of contaminating wild-type virus. The screening procedure and isolation of the recombinant viruses essentially followed the method of Summers and Smith (23). The resulting recombinant viruses from pVLα58, pVLα59, and pVLβ were designated as the α58 virus, α59 virus, and β virus, respectively.

Analysis of Recombinant Proteins in Sf9 Cells. Sf9 cells were cultured in TNM-FH medium (Sigma) supplemented with 10% fetal bovine serum at 27°C either as monolayers or in suspension in spinner flasks (Techne). To produce recombinant proteins, Sf9 cells seeded at a density of 10^6 cells per ml were infected at a multiplicity of 5–10 with recombinant viruses when the α58, α59, or β virus was used alone. The α and β viruses were used for infection in ratios of 1:10–10:1 when producing the prolyl 4-hydroxylase tetramer. The cells were harvested 72 hr after infection, homogenized in 0.01 M Tris, pH 7.8/0.1 M NaCl/0.1 M glycine/10 μM dithiothreitol/0.1% Triton X-100, and centrifuged. The resulting supernatants were analyzed by SDS/10% PAGE or nondenaturing 7.5% PAGE and assayed for enzyme activities. The cell pellets were further solubilized in 1% SDS and analyzed by SDS/10% PAGE. The cell medium at 24–96 hr postinfection was also analyzed by SDS/10% PAGE to identify any secretion of the resultant proteins into the medium. The cells in these experiments were grown in TNM-FH medium without serum.

When the time course of protein expression was examined, Sf9 cells infected with recombinant viruses were labeled with [^{35}S]methionine (10 μCi/μl; Amersham; 1 Ci = 37 GBq) for 2 hr at various time points between 24 and 50 hr after infection and collected for analysis by SDS/10% PAGE. To determine the maximal accumulation of recombinant protein, cells were harvested at various times from 24 to 96 hr after infection and analyzed by SDS/10% PAGE. Both the 0.1% Triton X-100- and 1% SDS-soluble fractions of the cells were analyzed.

Other Assays. Prolyl 4-hydroxylase activity was assayed by a method based on the decarboxylation of 2-oxo[1-^{14}C]glutarate (24). 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 (25). Protein disulfide-isomerase activity of the β subunit was measured by glutathione:insulin transhydrogenase assay (26). Western blot analysis was performed using a monoclonal antibody, 5B5, to the β subunit of human prolyl 4-hydroxylase (27).

Prolyl 4-hydroxylase was purified by a procedure consisting of poly(L-proline) affinity chromatography, DEAE-cellulose chromatography, and gel filtration (28).

Endoglycosidase H treatment (0.02 unit/ml) was carried out in 0.2 M sodium acetate, pH 5.8/0.1% SDS/1% 2-mercaptoethanol at 37°C for 2 hr.

RESULTS

Expression of the β Subunit of Human Prolyl 4-Hydroxylase in Sf9 Cells. The recombinant β virus was subjected to three rounds of plaque purification to generate a form entirely free of contaminating wild-type virus. The Sf9 cells were then infected with the purified virus. Metabolic 2-hr pulse-labeling experiments with [^{35}S]methionine indicated that the maximal production of recombinant protein occurred about 30–36 hr after infection, but that accumulation of the expressed protein continued until 72 hr (details not shown). The medium was then analyzed by SDS/PAGE followed by Coomassie staining or Western blotting with a monoclonal antibody, 5B5 (27), to human β subunit at 24–96 hr after infection and the cells at 72 hr. No band corresponding to the β subunit was found in the medium samples (data not shown), whereas a strong band was found in the 0.1% Triton X-100-soluble proteins of the cell homogenate (shown by Coomassie staining in Fig. 1). Only trace amounts of the β subunit remained in the 0.1% Triton X-100-insoluble pellet and were solubilized by 1% SDS. The maximum expression obtained was about 10 μg of recombinant protein per ml of cell culture.

Since the β subunit also functions as a protein disulfide-isomerase, the recombinant β-subunit polypeptide present in the 0.1% Triton X-100-soluble protein was assayed for the isomerase activity. After correction of the value for the proportion of the β subunit, the specific activity was the same as that of purified chick protein disulfide-isomerase (data not shown).

Expression of the α Subunits of Human Prolyl 4-Hydroxylase in Sf9 Cells. The recombinant viruses α58 and α59, coding for the two alternatively spliced forms of the α subunit (4), were plaque purified and used to infect Sf9 cells as above. Metabolic labeling experiments with [^{35}S]methionine indicated that the time courses for the production and accumulation of the two types of α-subunit polypeptide were essentially the same as for the β subunit (data not shown). The medium and cells were then analyzed as above by SDS/PAGE followed by Coomassie staining. As in the case of the β subunit, no secretion of the α-subunit polypeptides into the culture medium was found, although the α subunits have no Lys-Asp-Glu-Leu (KDEL) retention signal for endoplasmic reticulum luminal proteins (4, 29). In contrast to the data obtained with the β subunit, only trace amounts of the α subunits were solubilized from the cell homogenate with 0.1% Triton X-100, and only small amounts even with 1% Triton X-100; treatment with 1% SDS was needed to release the α-subunit polypeptides (Fig. 1). The maximum expression obtained for both types of α subunit was very similar to that of the β subunit, about 10 μg of recombinant protein per ml of cell culture.

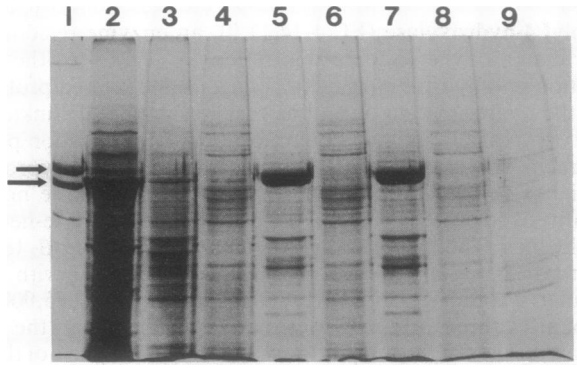

FIG. 1. SDS/PAGE analysis of the expression of the α and β subunits of prolyl 4-hydroxylase in Sf9 cells. Lane 1, purified chick prolyl 4-hydroxylase (the α and β subunits of which are shown by short and long arrows, respectively); lanes 2, 4, 6, and 8, 0.1% Triton X-100-soluble protein samples from Sf9 cells infected with the β, α58, and α59 virus and from mock-infected cells, respectively. After treatment with 0.1% Triton X-100, the cell pellets were solubilized with 1% SDS and protein samples obtained are shown in lanes 3, 5, 7, and 9 in the same order.

The α-subunit polypeptide has been found to exist in many cell types in two slightly divergent forms that differ in their carbohydrate content (30). The larger α subunit contains two N-linked high-mannose oligosaccharides, each with eight mannose units, whereas the smaller α subunit contains a single N-linked oligosaccharide with seven mannose units (30). Both types of cDNA that code for the α subunit, PA-58 and PA-59, encode two potential N-glycosylation sites, neither of which is in the alternatively spliced region (4), and the relationship between the two differentially glycosylated forms of the α subunit and the alternative splicing of the RNA transcripts has therefore remained unknown. No differences were found here in the mobilities of the polypeptide products of the α58 and α59 viruses in SDS/PAGE (Figs. 1 and 2). Partial endoglycosidase H treatment further demonstrated that both polypeptides were N-glycosylated to the same extent (Fig. 2). The data thus indicate that the two differentially glycosylated forms of the α subunit bear no relation to the two alternatively spliced forms of the RNA transcripts.

No prolyl 4-hydroxylase activity was found when the α subunits were expressed alone, without the β subunits, and the 1% SDS extracts of the cell homogenates were assayed for the enzyme activity under conditions in which the SDS was diluted to 0.001%, a concentration that in itself has no effect on the enzyme activity.

Expression of Fully Active Forms of Prolyl 4-Hydroxylase Tetramer in Sf9 Cells. To study whether it is possible to achieve association of the α and β subunits into an active prolyl 4-hydroxylase tetramer, Sf9 cells were coinfected with both the α and β viruses. These infections were initiated at the same time in most experiments, but in some the β-virus infection was begun 24 hr before the α-virus infection. The following combinations of the three types of virus were tested: α58/β, α59/β, and α58/α59/β. The ratio of multiplicity of infection with the α and β viruses varied from 1:10 to 10:1.

The polypeptide products of both types of α virus associated with the β subunit and formed an enzyme tetramer, as shown by nondenaturing PAGE analysis 72 hr after infection (Fig. 3). No differences were found in the association of the different α/β subunit combinations into the tetramer (i.e., α58/β, α59/β, α58/α59/β). Considerable amounts (up to half) of the α and β subunits remained unassociated in all the experiments (Fig. 3), and the maximal amount of enzyme tetramer produced appeared to be quite constant, about 5 μg per ml of cell culture, being independent of the ratio of infection with the α and β viruses (data not shown).

The enzyme tetramers resulting from infection with the viruses α58/β and α59/β were purified to homogeneity (Fig. 4) by an affinity column procedure (24, 28). No differences were found in the maximal velocities obtained with the two types of enzyme tetramer; both gave a velocity of about 12.5–13 mol/sec per mol of enzyme. This value agrees well

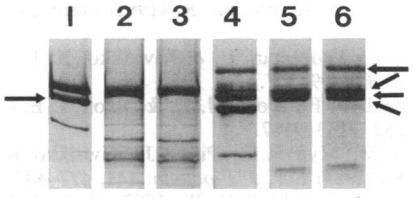

FIG. 2. Analysis of the carbohydrate moieties of recombinant α subunits by partial endoglycosidase H treatment. Lane 1, purified chick prolyl 4-hydroxylase; lane 4, chick prolyl 4-hydroxylase after endoglycosidase H treatment; lanes 2 and 3, 1% SDS soluble protein fractions from cells infected with the α58 and α59 virus, respectively; lanes 5 and 6, the same samples after endoglycosidase H treatment. The β subunit is shown by a long arrow on the left and the different forms of the α subunit are shown by short arrows on the right. Endoglycosidase H is shown by a long arrow on the right.

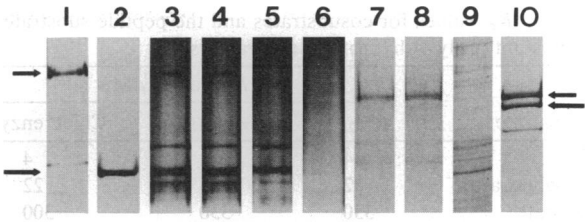

FIG. 3. Nondenaturing PAGE and SDS/PAGE analysis of the expression of the prolyl 4-hydroxylase tetramer in Sf9 cells. The samples in lanes 1–6 were run in a nondenaturing 7.5% polyacrylamide gel. Lane 1, purified chick prolyl 4-hydroxylase; lane 2, purified chick prolyl 4-hydroxylase dissociated into monomers by incubation in the presence of 1 mM dithiothreitol at 37°C for 2 hr; lanes 3–5, 0.1% Triton X-100-soluble protein fractions from Sf9 cells infected with α58/β, α59/β, or wild-type viruses, respectively; lane 6, 0.1% Triton X-100-soluble proteins from mock-infected Sf9 cells. After treatment with 0.1% Triton X-100, the cell pellets were solubilized with 1% SDS and run in an SDS/10% polyacrylamide gel (lanes 7–10). Lanes 7–9, protein samples from cells infected with α58/β and α59/β viruses and from mock-infected cells, respectively; lane 10, purified chick prolyl 4-hydroxylase. Locations of the prolyl 4-hydroxylase tetramer and monomers in the nondenaturing gel are shown on the left by a short and a long arrow, respectively. The α and β subunits in the SDS gel are indicated on the right by a short and a long arrow, respectively.

with that reported for the chick enzyme tetramer, which consists of two differentially glycosylated forms of the α subunit (24, 28). The K_m values for Fe^{2+}, 2-oxoglutarate, ascorbate, and the peptide substrate were also identical for the two types of recombinant enzyme tetramer and the chick enzyme tetramer (Table 1).

DISCUSSION

Although the structure of the prolyl 4-hydroxylase $\alpha_2\beta_2$ tetramer has been known for about 20 years (17, 18), all attempts to associate an active enzyme from the dissociated subunits *in vitro* have been unsuccessful (3, 19, 20). We report here the production of a fully active prolyl 4-hydroxylase tetramer in Sf9 insect cells using baculovirus vectors. The recombinant enzyme tetramer was indistinguishable from that isolated from vertebrate tissue in terms of its catalytic properties. Baculovirus expression would thus seem to be an excellent system for mass production of the enzyme (e.g., for crystallographic purposes). This system will also make it possible to initiate investigations into the effects of various mutations introduced into the α- or β-subunit coding sequences on the activity and other properties of the resulting enzyme tetramer.

When the β subunit was expressed alone, the polypeptide accumulated inside the cells, as in the case of the endogenous β subunit synthesized by a variety of vertebrate cells (1–3, 18). The retention signal for the endoplasmic reticulum luminal proteins in vertebrates is a carboxyl-terminal KDEL

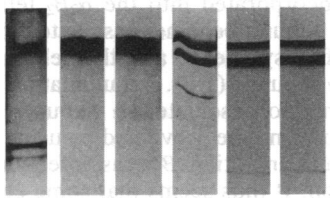

FIG. 4. Nondenaturing 7.5% PAGE (lanes 1–3) and SDS/10% PAGE (lanes 4–6) analysis of affinity column-purified recombinant prolyl 4-hydroxylase. Lanes 1 and 4, purified chick prolyl 4-hydroxylase; lanes 2 and 5 and lanes 3 and 6, purified prolyl 4-hydroxylase from Sf9 cells infected with α58/β or α59/β viruses, respectively.

Table 1. K_m values for cosubstrates and the peptide substrate of recombinant prolyl 4-hydroxylase tetramers

Substrate	K_m value, μM		
	$\alpha 58_2\beta_2$	$\alpha 59_2\beta_2$	Chick enzyme
Fe^{2+}	4	4	4
2-Oxoglutarate	22	25	22
Ascorbate	330	330	300
$(Pro\text{-}Pro\text{-}Gly)_{10}$	18	18	15–20

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. The values obtained are compared to those reported for chick enzyme in ref. 24.

sequence or its variant (29, 31), whereas yeast cells appear to recognize a carboxyl-terminal HDEL sequence (29). The present data indicate that the Sf9 insect cells can utilize the KDEL recognition signal.

We expected initially that the α subunit, when expressed alone, might be secreted into the medium, as it has no carboxyl-terminal KDEL sequence (4, 32), but no such secretion was detected. The reason for this lack of secretion appears to be the marked tendency of the α subunit to form insoluble aggregates in the absence of the β subunit (17, 18), as the use of 1% SDS was required to obtain an efficient extraction. The free α subunit thus appears to resemble the 88-kDa subunit of the microsomal triacylglycerol transfer protein complex, which also aggregates in the absence of the multifunctional protein disulfide-isomerase subunit (11). Further similarities between the α subunit and the 88-kDa subunit of the microsomal triacylglycerol transfer protein complex (11) are that neither of the polypeptides shows any biological activity in the absence of the multifunctional protein disulfide-isomerase subunit and neither of these polypeptides can be associated with the protein disulfide-isomerase *in vitro*. The β subunit thus appears to be necessary to keep the α subunit in a catalytically active, nonaggregated conformation. As the enzyme tetramer is a soluble endoplasmic reticulum luminal protein (1–3, 18), an additional function of the β subunit, due to its KDEL sequence, may be to retain the enzyme tetramer within this cell compartment.

The two alternatively spliced forms of the α subunit gave enzyme tetramers with identical catalytical properties, thus excluding the possibility that it might be necessary for the prolyl 4-hydroxylase tetramer to contain one α subunit of each kind (2, 4). The biological significance of the two alternatively spliced forms of the α subunit, if any, thus remains unexplained.

The accumulation of considerable amounts of the nonassociated α subunit in double infection with the α and β viruses differs distinctly from the situation in a number of cell types that produce an endogenous prolyl 4-hydroxylase tetramer (1–3, 18). The β subunit is usually synthesized in excess and enters a pool of the β subunit (protein disulfide-isomerase) before being incorporated into the $\alpha_2\beta_2$ tetramer (1–3). In contrast, the α subunit becomes associated into the tetramer immediately after synthesis, and the cells have no pool of unassociated α subunits (1–3). Accumulation of considerable amounts of the nonassociated α subunit in the present experiments could not be prevented by using the β virus in a large excess and initiating β-virus infection 24 hr before α-virus infection. It thus seems that some cellular system—e.g., a chaperone protein—may be needed to assist in the formation of the enzyme tetramer and that the rate of production of the recombinant α subunit exceeds the capacity of this system. The involvement of a chaperone would also explain the inability of the native α and β subunits to form an enzyme tetramer *in vitro*. The baculovirus expression system seems to offer an excellent tool for further investigations into the mechanisms involved in the association process.

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