Expression of Enzymatically Active Rat Dipeptidyl Peptidase IV in Chinese Hamster Ovary Cells after Transfection†

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ABSTRACT: Dipeptidyl peptidase IV (DPPIV) is a cell surface membrane glycoprotein expressed in many tissues. We have subcloned the coding region of a full-length cDNA for DPPIV into the inducible eukaryotic expression vector pMSG. The resulting construct was used to transfect Chinese hamster ovary (CHO) cells. Stable transformants were found to express DPPIV, and the expression is enhanced by dexamethasone. Metabolic labeling of the transfected cells with [^{35}S]Met followed by immunoprecipitation revealed the presence of two specific products of apparent M_r 100 000 (100-kDa form) and 110 000 (110-kDa form), respectively. Pulse–chase experiments demonstrated that the 100-kDa form can be chased into the 110-kDa form, suggesting the 100-kDa form is the precursor of the 110-kDa form. Further studies with endo H treatment demonstrated that the carbohydrate structures are of the high-mannose type, and of the complex type for the 100- and 110-kDa forms, respectively. The 110-kDa form is present at the cell surface as shown by its accessibility to cell surface iodination. The DPPIV expressed on the cell surface is resistant to digestion by relatively high concentrations of trypsin. Studies also demonstrated that the surface DPPIV is fairly stable with a half-life for turnover of about 40 h. Furthermore, the DPPIV produced in the transfected cells displays specific dipeptidyl peptidase activity. The stably transfected cells that express enzymatically active DPPIV in an inducible manner will provide an excellent system for further biochemical, functional, and cell biological characterizations of DPPIV.

Dipeptidyl peptidase IV is an exopeptidase found on the cell surface of many epithelial cells, endothelial cells, and some lymphocytes (Becker et al., 1986; Hubbard et al., 1985; Mentlein, 1988). DPPIV is a unique peptidase since it cleaves X-proline dipeptides from the N-termini of polypeptides. Although DPPIV has been extensively studied, the physiological roles played by DPPIV have not been fully revealed. Due to its unique specificity toward proline, DPPIV has been postulated to be important in the maturation and degradation of peptide hormones and neuropeptides (Mentlein, 1988), including substance P and interleukin II. High levels of DPPIV in the brush border of small intestine and kidney proximal tubules suggests that DPPIV is important for the metabolism and uptake of proline-containing peptides in these tissues (Miyamoto et al., 1987). Since DPPIV is expressed mainly in a subpopulation of T-lymphocytes (mostly T-helper cells), studies have shown that DPPIV is an important constituent in the process of lymphocyte proliferation and activation (Gruber et al., 1988; Scholz et al., 1985; Schon et al., 1986, 1987). Furthermore, recent investigations have shown that DPPIV is involved in the interaction of cells with the extracellular matrix through its binding activity to collagen and fibronectin (Bauvois, 1988; Hanski et al., 1985, 1986, 1988; Neumeier et al., 1984). Recent studies also have demonstrated that antibodies against DPPIV can induce acute and severe proteinuria in rats and have defined DPPIV as a pathogenic antigen of passive nephritis (Mendrick & Rennke 1988a,b; Natori et al., 1987), further suggesting that DPPIV plays a critical part in maintaining normal tissue structure. In order to further define the functional properties of DPPIV, it will be of significance to have stably transfected cells that express DPPIV in an inducible manner.

We have recently isolated a full-length cDNA clone for a membrane glycoprotein with an apparent M_r of 110000 (Hong & Doyle, 1987). Recent studies have demonstrated that the cloned cDNA is for dipeptidyl peptidase IV (Hong et al., 1989). In this report, we have used a eukaryotic expression vector to express DPPIV in stably transfected Chinese hamster ovary cells and have focused attention on the biogenesis of DPPIV in the transfected cells. The results demonstrate that DPPIV is transported normally to the cell surface. Importantly, the expressed protein has DPPIV enzymatic activity. This study also reveals some important biochemical properties of DPPIV in the transfected cells. The expression of enzymatically active DPPIV in stably transfected CHO cells provides a system for more detailed biochemical, cell biological, and especially functional studies of DPPIV.

Materials and Methods

Materials. Na^{125}I and [^{35}S]methionine (800 Ci/mmol) were obtained from Amersham Corp. and Dupont NEN, respectively. Protein A–Sepharose 4B and the eukaryotic expression vector pMSG were purchased from Pharmacia. Endoglycosidase H was from Miles Laboratories. Rabbit reticulocyte lysates were from Promega. Nitrocellulose paper was from Schleicher & Schuell. Restriction enzymes and T4 DNA ligase were from Promega or BRL. DMEM culture media and fetal bovine serum were from Gibco. Cellulose acetate sheets impregnated with Ala-Pro-AFC [amino-4-(trifluoromethyl)coumarin] for DPPIV enzyme overlay were from Enzyme Systems. Other reagents and chemicals were from Sigma and Fisher.

Plasmid Construction for Expression of DPPIV. As shown in Figure 1, a 3.2-kb *Eco*RI fragment derived from the λNC_4

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clone (Hong & Doyle 1987, 1988) which is a full-length cDNA clone isolated from a rat kidney λgt11 cDNA library was first subcloned into the in vitro transcription vector pGEM-4Z to generate pG4Z/gp110. The pG4Z/gp110 is used for in vitro transcription to make DPPIV mRNA transcripts (Hong & Doyle, 1988). The pG4Z/gp110 was cut with the restriction enzyme *Eco*RI, and the DNA fragments were then converted to blunt ends as described (Davis et al., 1986). After digestion with restriction enzyme *Xho*I, the *Eco*RI (blunt)–*Xho*I fragment containing all the coding sequence for DPPIV was gel-purified. The eukaryotic expression vector pMSG (Lee et al., 1981) was double-digested with *Sma*I and *Xho*I. *Sma*I restriction enzyme digestion results in blunt ends, which is compatible with *Eco*RI (blunt). The *Eco*RI (blunt)–*Xho*I fragment was inserted into the *Sma*I and *Xho*I sites of pMSG to get pMSG/gp110.

Transfection of CHO Cells and Selection of Stable Transformants. The protocol described by Davis et al. (1986) was followed for transfection. Briefly, about 10^6 cells were incubated with $CaPO_4$/DNA precipitate containing 20 μg of supercoiled pMSG/gp110 plasmid DNA. After 4-h incubation at 37 °C, the cells were washed and glycerol-shocked for 5 min at room temperature. Cells were washed and incubated at 37 °C for 24 h. Cells were then trypsinized and selected for *Escherichia coli* gpt in DMEM supplemented with 5% FBS, 250 μg/mL xanthine, 25 μg/mL mycophenolic acid, 0.5 μg/mL aminopterin, 10 μg/mL thymidine, and 15 μg/mL hypoxanthine. After 3-weeks selection, all the transformants were pooled for studies of the expression of DPPIV in a population of cells.

Immunoblot Analysis. Stably transfected cells were cultured in the presence of 0.1 μM dexamethasone for induction in all the induction experiments. Cells (either induced or not) were washed in hypotonic solution (buffer A) containing 10 mM Hepes, pH 7.2, 0.25 M sucrose, and 1 mM PMSF. The washed cell pellets were extracted with extraction buffer [1% Triton X-100, 0.2% SDS, 150 mM NaCl, 5 mM EDTA, 50 mM Tris (pH 7.4), 1 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL antipapain, and 10 μg of pepstatin]. After sonication, the extracts were clarified of cell debris by centrifugation for 30 min in a microfuge. Aliquots of cell extracts were mixed with an equal volume of 2× SDS sample buffer and resolved by SDS–PAGE. Transfer of proteins from gel to nitrocellulose filter, incubation with antibodies against DPPIV, and incubation with ^{125}I protein A were as described (Hong et al., 1988).

Isolation and Analysis of RNA. The standard protocol (Davis et al. 1986; Maniatis et al., 1982) was followed and modified as follows: Cells were scraped off tissue culture dishes after being washed with PBS. Cells were resuspended in 10 mM Tris (pH 8.5), 1.5 mM $MgCl_2$, and 140 mM NaCl. NP-40 was added to a final concentration of 0.2%. Nuclei were removed by centrifugation at 2000*g* for 10 min at 4 °C. The supernatant fraction was mixed with one-fifth volume of 50 mM Tris (pH 8.0), 10 mM EDTA, 10 mM NaCl, and 0.5% SDS. After two extractions with equal volumes of phenol–chloroform–isoamyl alcohol (PCI), the aqueous phase was adjusted with one-tenth volume of 3 M sodium acetate, pH 5.0, and 2.5× volumes of ethanol. RNA was precipitated overnight at −20 °C. Twenty micrograms of total RNA was denatured with glyoxal and resolved in a 10 mM sodium phosphate–agarose gel (pH 6.7). After RNA transfer to a nitrocellulose filter, the nitrocellulose filter was treated by boiling in a solution of 20 mM Tris (pH 8.0). Prehybridization and hybridization were done as before (Hong et al., 1989).

Metabolic Labeling of Cell Cultures. Monolayer cells (either induced or not) were preincubated in Met-free DMEM culture media for 1 h. After being washed once with DMEM minus Met, cells were labeled with [^{35}S]Met. See figure legends for amounts of [^{35}S]Met, length of labeling, and chase with excess unlabeled methionine, if any.

Immunoprecipitation. Labeled cells were washed with PBS and then with buffer A. After solubilization in extraction buffer, extracts were clarified of cell debris by centrifugation at 4 °C for 30 min in a microfuge, and 5 μL of antiserum to DPPIV was added. After incubation at room temperature for 2 h (or overnight in a cold room), 8 mg of protein A–Sepharose was added. After 1-h incubation at room temperature, the protein A–Sepharose was washed 4 times with 50 mM Tris (pH 7.4), 1% Triton X-100, 0.2% SDS, 150 mM NaCl, and 1 mM PMSF, and then washed 3 times with 20 mM Tris (pH 7.4), 150 mM NaCl, and 1 mM PMSF. Immuniprecipitates were then eluted by boiling for 4 min in either 1× SDS sample buffer for gel electrophoresis or in 20 mM Tris (pH 7.4), 150 mM NaCl, 10 mM DTT, and 1% SDS (elution buffer) for endoglycosydase H (endo H) treatment.

Endo H Treatment. Immunoprecipitates in elution buffer were diluted (1:10) with 150 mM sodium citrate, pH 5.0, and 2 milliunits of endo H was added. After incubation overnight at 37 °C, trichloroacetic acid (TCA) was added to a final concentration of 10%. TCA precipitates were washed with acetone and then ethanol, and were dissolved in SDS sample buffer.

Cell Surface Iodination. Cell monolayers in tissue culture dishes (100 × 20 mm, Falcon) were washed once with PBS and once with Hanks' buffer; 2.6 mL of Hanks' buffer, 0.26 mL of 1 M Tricine, pH 7.0, 0.054 mL of lactoperoxidase (1 mg/mL), 0.054 mL of 0.5 M glucose, and 0.008 mL of $Na^{125}I$ (80 μCi) were added to each dish. Iodination was initiated by adding 0.025 mL of glucose oxidase (1 mg/mL) to each dish. Iodination was carried out on ice for 45 min.

DPPIV Enzyme Assay. DPPIV activity was identified enzymatically after separation by SDS–PAGE in the presence of 0.1% SDS by overlaying the unfixed gel for 30 min at 37 °C with a sheet of cellulose acetate impregnated with the fluorogenic substrate Ala-Pro-AFC (Smith, 1984).

Results

Expression of DPPIV in Transfected CHO Cells. In order to establish cells that are transfected with DPPIV cDNA, we sought to express DPPIV in CHO cells because antibodies to DPPIV do not recognize endogenous protein of CHO cells as assessed by immunoblot analysis (see below) and CHO cells can be readily transfected with the inducible expression vector pMSG. The *E. coli* gpt gene serves as a selection marker for cells that are transfected (Lee et al., 1981). As shown in Figure 1, the coding sequence for DPPIV was inserted into the *Sma*I and *Xho*I sites of the polylinker region of pMSG so that expression of DPPIV is under the control of the mouse mammary tumor virus long terminal repeat (MMTV LTR), which is activated by the glucocorticoid receptor in the presence of dexamethasone. Figure 2A shows an immunoblot analysis of transfected CHO cells (lanes 3–5) and control CHO cells (lanes 1–2) in the presence of 0.1 μM (lane 4) and 0.5 μM (lanes 2 and 5) dexamethasone. As can be seen, control CHO cells do not express DPPIV in the presence or absence of dexamethasone, whereas DPPIV in transfected cells is induced by dexamethasone (comparing lane 3 to lanes 4 and 5); 0.1 μM dexamethasone resulted in as much induction as 0.5 μM, suggesting that 0.1 μM produces maximal induction. Therefore, 0.1 μM dexamethasone was used in all the following

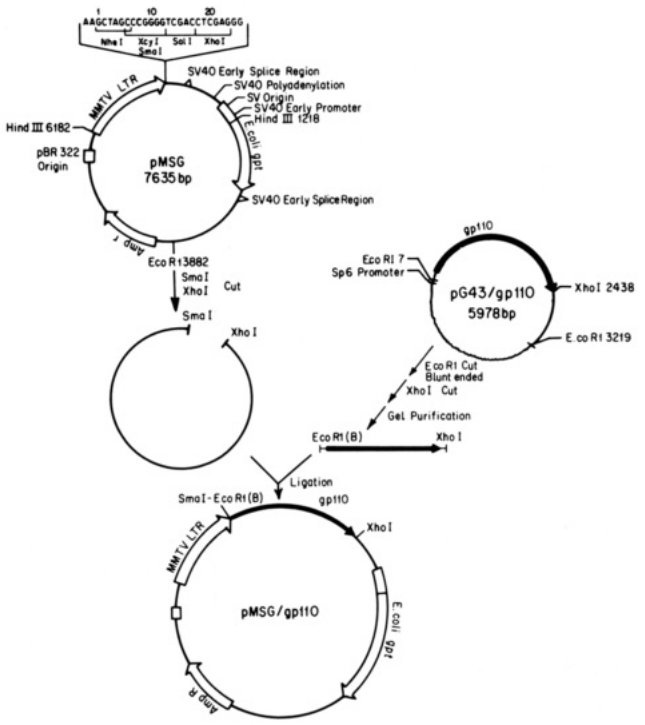

FIGURE 1: Plasmid construct for DPPIV expression. The coding region for DPPIV from plasmid pG-4Z/gp110 (27) was subcloned into the eukaryotic expression vector pMSG so that the expression of DPPIV is under the control of MMTV LTR transcriptional elements.

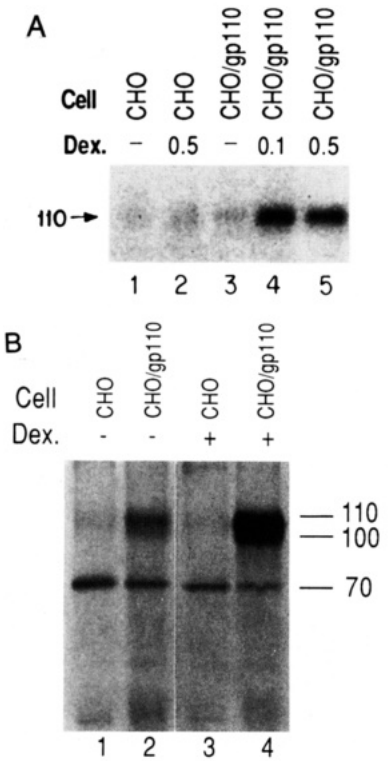

FIGURE 2: Expression of DPPIV in transfected cells. As assayed by immunoblot of cell extracts (A) and immunoprecipitation from [^{35}S]Met-labeled cells (B), DPPIV is expressed in transfected cells but not in control cells. The expression of DPPIV was induced by dexamethasone. Two forms of M_r 100000 and 110000, respectively, were precipitated specifically (lane 4, panel B). As demonstrated below, the 100-kDa form is the precursor of the 110-kDa form.

induction experiments. The expression for DPPIV was also assessed by immunoprecipitation of metabolically [^{35}S]Met-labeled cells as shown in Figure 2B. There is no protein that is specifically immunoprecipitated with anti-DPPIV antibodies

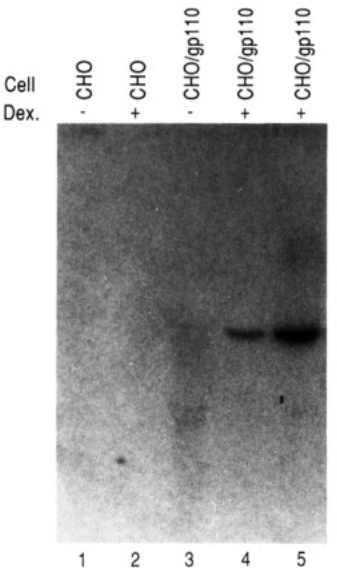

FIGURE 3: Northern blot analysis of total RNA (20 μg) from transfected (lanes 3–5) and control (lanes 1 and 2) cells in the presence (lanes 2, 4, and 5) or absence (lanes 1 and 3) of dexamethasone for 4 h (lane 4) or overnight (lanes 2 and 5).

from control CHO cells whether the cells are induced (lane 3) or not (lane 1) with dexamethasone. The 70-kDa polypeptide which is present in all of the immunoprecipitates is due to nonspecific binding since it is also precipitated with preimmune serum (data not shown). In transfected cells, two proteins (of M_r 100000 and 110000, respectively) are specifically immunoprecipitated. These two proteins are induced in the presence of dexamethasone (comparing lane 4 to lane 2). As shown below in biogenesis, the 100-kDa form represents the precursor of the mature 110-kDa DPPIV.

Induction of DPPIV by Dexamethasone Is Due to an Increase in the mRNA Level. Since the expression of DPPIV is under the control of MMTV LTR cis-acting elements for transcription, the elevated protein level of DPPIV due to the presence of dexamethasone as assessed by both immunoblot and immunoprecipitation analysis is most likely due to increased transcription of the DPPIV coding sequence. Northern blot analysis (Figure 3) was used to detect the steady-state level of DPPIV mRNA in total RNA isolated from control CHO cells (lanes 1 and 2) and transfected cells (lanes 3–5). The probe used was a *Bgl*II fragment from the DPPIV coding region of about 1.9 kb that was labeled through random primer labeling (Feinberg & Vogelstein, 1983). No mRNA for DPPIV was detected in control CHO cells in the presence (lane 2) or absence (lane 1) of dexamethasone. A low level of DPPIV mRNA was present in transfected cells in the absence of dexamethasone (lane 3). The mRNA level for DPPIV was increased about 5- and 15-fold in transfected cells after 4 h (lane 4) and overnight dexamethasone induction (lane 5), respectively. These results demonstrate that the increased DPPIV protein level in the presence of dexamethasone is due to an increased steady-state DPPIV mRNA level, which is most likely due to increased transcription of the DPPIV coding sequence.

Biogenesis of DPPIV in Transfected Cells. As seen in Figure 2, antibodies against DPPIV specifically precipitate two polypeptides (100 and 110 kDa, respectively) from the transfected cells. In order to reveal the relationship between the 100- and 110-kDa forms, and to gain insight into the biogenesis of DPPIV in transfected cells, we performed a pulse–chase experiment with transfected cells induced with dexamethasone. Cells were pulse-labeled for 20 min with

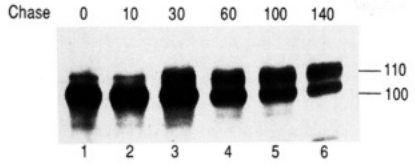

FIGURE 4: Biogenesis of DPPIV in transfected cells. Cells were pulse-labeled with [^{35}S]Met for 20 min and chased for various time (in minutes) as indicated. The cell extracts were processed for immunoprecipitation, SDS–PAGE, and autoradiography. The 100-kDa form is the precursor of the 110-kDa form since the former can be chased into the latter. About 50% of the 100-kDa form is chased into the 110-kDa form in about 120 min.

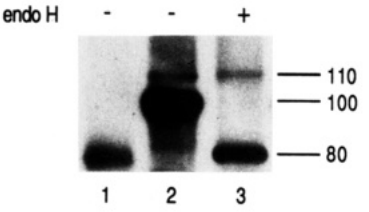

FIGURE 5: Endo H digesion of DPPIV immunoprecipitated from transfected cells. As seen, the 100-kDa form can be converted into an 80-kDa form by endo H digestion, while the 110-kDa form is resistant to endo H treatment. This result shows that the carbohydrate in the 100- and 110-kDa forms is of the high mannose and complex type, respectively. The product of in vitro translation of RNA derived from DPPIV cDNA in the absence of membranes is shown in lane 1 and has an apparent molecular weight of 80 000.

[^{35}S]Met and then chased with excess unlabeled Met for various times as indicated in Figure 4. At all time points, cells were processed for immunoprecipitation with antibodies to DPPIV. The immunoprecipitates were then analyzed by SDS–PAGE and autoradiography. As can be seen, the 100-kDa form is the major form detected in the pulsed cells. As the chase proceeds, the amount of the 100-kDa form decreases whereas the amount of the 110-kDa form increases, demonstrating that the 100-kDa form was being converted to the 110-kDa form during the chase. Fifty percent of the 100-kDa form was converted to the 110-kDa form within about 120 min. We interpret that the 100-kDa form is the precursor for the biogenesis of DPPIV and the 110-kDa form is the mature protein. If the 100-kDa form is a precursor in the biogenesis of DPPIV, it may contain only high mannose type carbohydrate chains. Similarly, if the 110-kDa form is the mature protein, the carbohydrate chains on the protein should have been processed to the complex type. To determine if this is the case, immunoprecipitates from labeled cells containing both forms were subjected to treatment with endo H as shown in Figure 5. Endo H converted the 100-kDa form into a polypeptide of apparent M_r 80 000, while the 110-kDa form is endo H resistant (lane 3). These results further support our interpretation that the 100-kDa form is a precursor of and is processed to the 110-kDa mature form of DPPIV. Lane 1 shows the immunoprecipitate with antibodies to DPPIV from an in vitro translation reaction with a mRNA template transcribed in vitro from the full-length cDNA clone. A protein of apparent M_r 80 000 was produced. The similar sizes of the endo H converted form and the protein produced from in vitro translation suggest that there is no detectable O-linked glycosylation on the DPPIV protein in the transfected cells.

Cell Surface Expression of DPPIV. As shown above, the 110-kDa form is the mature form of the 100-kDa precursor during the biogenesis of DPPIV. Since DPPIV is a cell surface glycoprotein, it is important to determine if DPPIV is transported to the cell surface in the transfected cells. Transfected cells either induced with dexamethasone or not induced were

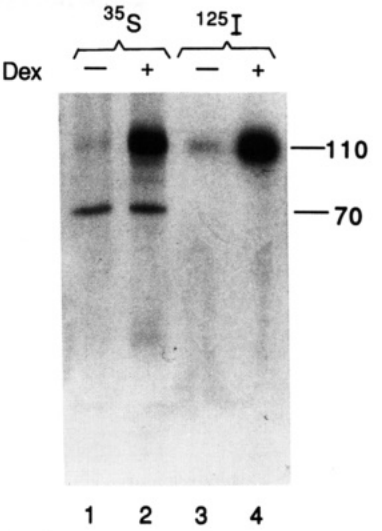

FIGURE 6: Cell surface expression of DPPIV. The 110-kDa but not the 100-kDa form can be surface-labeled with ^{125}I through a lactoperoxidase-catalyzed reaction (lanes 3 and 4), suggesting that the 110-kDa but not the 100-kDa form has been transported to the cell surface. The cells were induced either with (lane 4) or without (lane 3) dexamethasone before iodination. Shown in lanes 1 and 2 are the proteins immunoprecipitated from [^{35}S]Met-labeled cells in the presence (lane 2) or absence of dexamethasone.

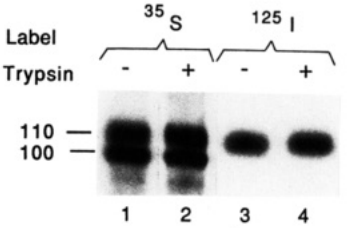

FIGURE 7: Trypsin resistance of surface DPPIV. Cells were labeled with [^{35}S]Met (lanes 1 and 2) or ^{125}I and then treated with (lanes 2 and 4) or without (lanes 1 and 3) trypsin at 1 mg/mL on ice for 60 min. Extracts from these cells were subjected to immunoprecipitation, SDS–PAGE, and autoradiography.

subject to cell surface iodination catalyzed by lactoperoxidase. The iodination was performed on ice to prevent any further membrane trafficking so that only cell surface proteins are labeled. The iodinated cells were processed for immunoprecipitation, and the immunoprecipitated proteins were analyzed by SDS–PAGE and autoradiography as shown in Figure 6. In both induced (lane 4) and noninduced cells (lane 3), the 110-kDa form was iodinated and specifically precipitated by antibodies to DPPIV. As shown, dexamethasone induction also increases cell surface expression of DPPIV in the transfected cells. Taken together, the above data demonstrate that transfected cells can express and process DPPIV, and transport the processed DPPIV to the cell surface.

Trypsin Resistance of Surface DPPIV. In an attempt to selectively remove surface DPPIV, we found that DPPIV expressed on the cell surface is fairly resistant to trypsin digestion. When the transfected cells were labeled with [^{35}S]-Met, we expected that the 100-kDa precursor form of DPPIV would be resistant to trypsin since it is localized intracellularly (based on the high mannose type structure of its carbohydrate chains and its inaccessibility to surface iodination), whereas the 110-kDa form should be accessible to trypsin digestion. As shown in Figure 7, both the 100- and 110-kDa forms are resistant to trypsin (comparing lane 2 to lane 1). The trypsin resistance of the 110-kDa form can be explained by two alternatives: The first explanation is that surface DPPIV (110-kDa form) is resistant to trypsin (a property of the protein

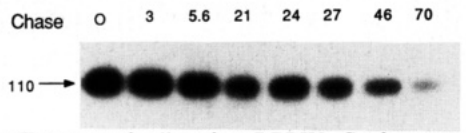

FIGURE 8: Turnover of cell surface DPPIV. Surface proteins were labeled with ^{125}I through lactoperoxidase. The same number (10^6) of cells were chased for various time as indicated (in hours). Extracts from these cells were then analyzed by immunoprecipitation, SDS–PAGE, and autoradiography.

itself). The other explanation is that only a small portion of the 110-kDa form is transported to the cell surface with the majority of the 110-kDa form localized intracellularly (e.g., Golgi and post-Golgi structures) and therefore not accessible to trypsin (location dependent and not an intrinsic property of the protein). In order to distinguish between these two possibilities, transfected cells were subjected to surface iodination on ice so that only cell surface localized proteins are labeled. If the latter explanation is right, trypsin should degrade all labeled DPPIV. If the trypsin resistance is an intrinsic feature of DPPIV, we should see that the surface-labeled DPPIV is also resistant to trypsin. As shown in lane 4, the majority of the surface-labeled DPPIV remains intact after 1-h trypsin digestion on ice (at a concentration of 1 mg/mL). These results suggest that the trypsin resistance of the cell surface DPPIV is an intrinsic feature of DPPIV.

DPPIV Is Stable in Transfected Cells. Expression of foreign proteins in transfected cells is useful for a detailed analysis of structure and function relationships of the foreign proteins using biochemical, cell biological, and molecular biological techniques. Many proteins have been expressed successfully in various cell types, but the stability of these proteins in the transfected cells has not been assessed in detail. Since protein turnover contributes also to the steady-state protein level, it was of importance to determine the turnover rate of the expressed protein in the transfected cells. To do this, transfected CHO cells (induced with dexamethasone) were surface labeled with ^{125}I via lactoperoxidase-catalyzed iodination. Labeled cells were cultured for various periods of time as indicated in Figure 8 and then subjected to immunoprecipitation with antibodies against DPPIV. The immunoprecipitates were resolved by SDS–PAGE and detected by autoradiography. As shown in Figure 8, DPPIV is very stable. After 46 h of culture, about 50% of the labeled protein is still intact. About 25–30% of the protein remains intact after 72-h incubation. The average $t_{1/2}$ estimated for DPPIV in the transfected cells is 40 h.

Discussion

The results presented here demonstrate that we have successfully expressed rat DPPIV in Chinese hamster ovary cells. DPPIV is an excellent endogenous marker for the biogenesis of membrane glycoproteins, since it is expressed on the cell surface of many cell types. The rate of biogenesis of DPPIV in the transfected cells is consistent with that reported for DPPIV in vivo (Bartles et al., 1987) with about 120 min required for the appearance of DPPIV on the cell surface. The similar time required for the biogenesis of DPPIV in the transfected CHO cells and in vivo in the liver suggests that CHO cells can be useful for a general characterization of the biogenesis of membrane proteins. The consistency in molecular weight of the in vitro produced protein and the product derived from endo H digesion of DPPIV produced in CHO cells suggests that the DPPIV produced in the transfected CHO cells does not contain any detectable O-linked carbohydrate. The resistance of cell surface DPPIV in the transfected cells to relatively high concentrations (1 mg/mL) of trypsin is consistent with and is important for its normal cellular localization and its identity as a membrane peptidase. Using in vitro transcription and translation systems, we have recently predicted that DPPIV is anchored in the membrane through its N-terminal noncleavable signal sequence with an N-terminal six amino acid residues on the cytoplasmic side of the membrane (Hong & Doyle, 1988). This predicted membrane orientation is in a good agreement with and further confirms previous studies from other laboratories (Macnair & Kenny, 1979; Semenza, 1986). This membrane orientation of DPPIV is similar to the orientations predicted for sucrase–isomaltase (Hunziker et al., 1985), for γ-glutamyltranspeptidase (Laperche et al., 1986), and for aminopeptidase N (Olsen et al., 1988), three other cell surface membrane enzymes. How and whether this membrane orientation is related to the cell surface expression of these proteins has not yet been answered. The successful expression of DPPIV in these stably transfected cells offers a potentially useful system for these types of studies.

The DPPIV encoded by the cloned cDNA in the transfected cells has dipeptidase enzymatic activity since the DPPIV immunoprecipitated from transfected cells can cleave a fluorogenic dipeptide substrate (Ala-Pro-AFC) (data not shown) and this activity is enhanced by dexamethasone. This not only further confirms the identity of the cDNA but also offers the potential for detailed studies on the structure–function relationship of this protein. DPPIV is a unique peptidase in terms of its specificity, and it will be interesting to learn more about its catalytic properties and the structural features that confer these properties. In combination with site-directed mutagenesis of the cDNA for enzymatically active DPPIV, the expression of DPPIV in the transfected cells will provide us with a novel experimental system for these further studies.

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Insertion of New Sequences into the Catalytic Domain of an Enzyme†

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ABSTRACT: Activities of enzymes can be modified by the replacement of active-site amino acids with residues that strengthen specific interactions with substrates or that alter the specificity. The scope for engineered enzymes would be broadened if additional, new sequences could be inserted into a catalytic domain. Properly designed, these sequences could encode new ligand binding sites, be intermediates in the construction of chimeric enzymes, or alter the internal flexibility and "breathing" modes of the active-site region. As a first step toward this objective, we inserted oligopeptides of up to 14 amino acids into various locations within an 82 amino acid region of the adenylate synthesis domain of *Escherichia coli* methionyl-tRNA synthetase. These sites include ones that are flanked by sequences that are conserved between the proteins from *E. coli* and the yeast *Saccharomyces cerevisiae* and those that are essential for activity and stability. We found that all of the insertional mutants are stable and some have catalytic parameters for adenylate synthesis that are comparable to those of the wild-type enzyme. Thus, such an approach may provide for a variety of novel applications.

Modification of enzyme activity to change substrate interactions or alter specificity has been achieved by site-directed mutagnesis of active-site amino acids. Broader applications for enzyme design include the insertion of new ligand binding sites, alteration of internal flexibility (Bone et al., 1989), and the construction of chimeras that combine desired traits of two or more enzymes. Such novel characteristics require the introduction of protein segments into enzyme domains. To explore new approaches to enzyme engineering, we chose to insert oligopeptides into a member of a major class of enzymes.

The aminoacyl-tRNA synthetases catalyze the aminoacylation of specific transfer RNAs. The two-step reaction involves condensation of amino acid with adenosine triphosphate (ATP) to produce an enzyme-bound aminoacyl adenylate intermediate and subsequent transfer of the amino acid to tRNA to give aminoacyl-tRNA (Schimmel, 1987):

$$\text{ATP} + \text{AA} \rightleftarrows \text{AA-AMP} + \text{PPi} \quad (1)$$

$$\text{AA-AMP} + \text{tRNA} \rightleftarrows \text{AA-tRNA} + \text{AMP} \quad (2)$$

Although they catalyze the same chemical reaction, the synthetases vary considerably in their primary sequences and gross structural features (for example, different subunit sizes and quaternary structures) (Schimmel, 1987). However, crystallographic analysis has determined that a domain containing a Rossmann nucleotide fold (Rossmann et al., 1975) is found in the amino-terminal portion of two synthetases: *Escherichia coli* methionyl-tRNA synthetase and *Bacillus stearothermophilus* tyrosyl-tRNA synthetase (Bhat et al., 1982; Risler et al., 1981; Zelwer et al., 1982; Blow & Brick, 1985; Brunie et al., 1987). Some structural similarity between parts of the nucleotide folds of these two enzymes has been noted. Amino acid and ATP are bound in this domain (Bhat et al., 1982; Risler et al., 1981; Zelwar et al., 1982; Blow & Brick, 1985; Brunie et al., 1987), which presumably is the site of amino acid activation. Sequence analysis and structural modeling suggest that a number of other synthetases share this motif (Schimmel, 1987; Starzyk et al., 1987).

The roughly 361 amino acid α/β structure that forms the nucleotide fold of *E. coli* methionyl-tRNA synthetase is depicted in Figure 1 (Barker et al., 1982; Dardel et al., 1984; Brunie et al., 1987). The exact disposition of methionine and ATP in this structure is not yet clarified (Brunie, et al., 1987). A segment designated "connective polypeptide 1" (CP1)

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