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Expression and Transport into Mitochondria of Bovine Cytochrome P-450(SCC) in Insect Cells Using the Baculovirus Expression System

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Summary: Bovine cytochrome P-450(SCC) introduced with the baculovirus host vector system was found to be expressed in *Spodoptera frugiperda* cells. Cell fractionation analysis indicated that the P-450(SCC) expressed as the precursor form was transported into mitochondria and converted to a mature form. However, this form did not exhibit definite activity for cholesterol side chain cleavage. These findings suggest that most of the P-450(SCC) expressed by this system is an inactive protein within mitochondria that is not folded to the conformation of the active enzyme and/or does not incorporate heme appropriately.

P-450(SCC) is a major protein component of mammalian steroidogenic mitochondria, and catalyzes the side chain cleavage of cholesterol, which is an initial and rate-limiting step in the biosynthesis of corticoids and sex hormones [1]. Because of its physiological importance and high content in mitochondria, P-450(SCC) has attracted attention in various fields of biology [2, 3, 4]. This enzyme has been shown to be synthesized on free polysomes as a larger precursor form, imported into mitochondria, and located on the matrix surface of the inner membrane after its conversion to the mature form by a processing protease [5, 6]. Furthermore, using cholesterol and several hydroxycholesterols as enzyme substrates, Shikita and Hall [7] determined the stoichiometry of the reaction, finding that the side chain cleavage of cholesterol involves three successive monooxygenase reactions at different sites of the substrate.

For detailed studies of the reaction mechanism and other characteristics of this enzyme, various analyses of proteins obtained by the site-directed mutations should be useful. But to obtain such proteins, a heterologous expression system that produces active P-450(SCC) in large amounts is indispensable. So far the heterologous expression of functional P-450(SCC) in the eukaryotic cells has been achieved only in COS cells transfected with a plasmid vector, and its expression in these cells was transient and too low to allow further analyses [8, 9].

The present paper describes an attempt to express bovine P-450(SCC) carried by a baculovirus vector in insect cells. P-450(SCC) was effectively produced and processed to a mature form in the mitochondria of the transfected cells, but its activity was not detected clearly.

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Materials and Methods

Spodoptera frugiperda Sf21 cells were supplied from Dr. K. Suzuki, Nippon Ciba-Geigy (Japan), and wild-type AcNPV(baculovirus) and pBlueBac were purchased from Invitrogen Co. (San Diego, U.S.A.). P-450 (SCC) cDNA isolated from a bovine adrenal cortex cDNA library [10] was a gift from Dr. T. Omura, Kyushu University. The cDNA was modified to construct the transfer vector as described in the legend to Fig.1. Two kinds of recombinant plasmids carrying the insert in the correct and reverse orientations were constructed, and used for cotransfection of wild-type baculovirus AcNPV DNA into Sf21 cells. The cells were maintained on TNM-FH-10% fetal bovine serum medium at 27°C [11]. Two kinds of recombinant viruses with opposite orientations were isolated by β-galactosidase assay, by the method of Vialard *et al.* [12] and were named AcNPV-scc(+), and AcNPV-scc(-), respectively.

To enhance the expression of P-450(SCC), hemin chloride (dissolved in 0.1 M NaOH) and an enzyme substrate, 20-α-hydroxycholesterol (dissolved in DMSO) were added to the culture medium at concentrations of 5μg/ml and 20mM, respectively. Sf21 cells were harvested at various times after infection, and washed with phosphate buffered saline. Then they were disrupted in a

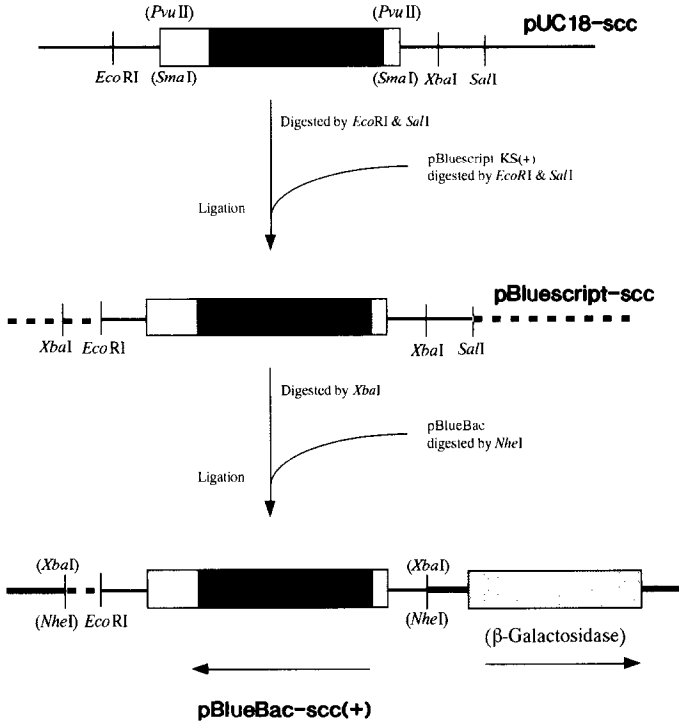

Fig. 1. Scheme for construction of P-450(SCC) transfer vector pBlueBac-scc(+), and -scc(-). The *Pvu*II fragment of cDNA for P-450(SCC) of bovine adrenal cortex was blunt-ended and inserted into the *Sma*I site of pUC18. The recombinant plasmid (named pUC18-scc) was then digested with *EcoR*I and *Sal*I. The fragment obtained was subcloned into pBluescript KS(+). The *Xba*I fragment derived from the recombinant plasmid, pBluescript-scc was isolated and inserted into the *Nhe*I site of the expression vector pBlueBac, because the vector cut with *Nhe*I can accept a DNA fragment digested with *Xba*I. The recombinant plasmids carrying the insert in the correct and reverse orientations were named pBlueBac-scc(+), and pBlueBac-scc(-), respectively.

The open boxes indicate the noncoding regions of P-450(SCC) cDNA, while the filled one indicates its coding region. The solid and dashed line indicate pUC18 and pBluescript KS(+), respectively. The thick solid line represents pBlueBac. The shaded box shows the β-galactosidase gene. Arrows below the map of pBlueBac-scc(+) indicate the directions of transcription.

Potter homogenizer with 0.25M sucrose-10mM Tris-HCl buffer(pH7.5) containing 0.2mM phenylmethylsulfonyl fluoride (PMSF), 10μg/ml leupeptin, and 10μg/ml pepstatin. The homogenate obtained was fractionated as described by Ito *et al* .[13].

The activity of P-450(SCC) was measured by normal-phase HPLC after cholesterol oxidase treatment [17] or by TLC with a radioactive substrate [18]. The assay was performed with the reconstituted system supplied with NADPH-adrenodoxin reductase and adrenodoxin purified from bovine adrenal cortex mitochondria as described in Mitani *et al* .[16]. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 9% gel [15]. Western blotting analysis was performed with rabbit polyclonal antibodies raised against bovine P-450(SCC)[16] and goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (BIO-RAD, U.S.A.). Anti-P-450(SCC) antibody, NADPH-adrenodoxin reductase and adrenodoxin were generously provided by Dr. F. Mitani, Keio University. Protein was determined by a modification of the method of Lowry *et al* .[14].

Results and Discussion

The expression of P-450(SCC) in Sf21 cells was examined by Western blotting at several times after infection of AcNPV-scc(+) virus. Two bands that reacted with anti-P-450(SCC) antibody appeared after 36 hours, indicating the expression of this enzyme (Fig.2). Based on their mobilities on gel, the upper and lower bands were concluded to be the precursor and the mature form of P-450(SCC), respectively. Their intensities increased further during culture. However, since the condition of the cells deteriorated after 48 hours, the cells were harvested 48 hours after infection in following experiments.

To determine the intracellular localization of the expressed P-450(SCC), we prepared four subfractions from a homogenate of the infected cells and analyzed each fraction by Western blotting. As shown in Fig.3, both the precursor and the mature form of P-450(SCC) expressed were mainly recovered in the mitochondrial fraction, and were not detected in the cytosolic fraction. A trace of P-450(SCC) was also detected in the microsomal fraction. The purity of the

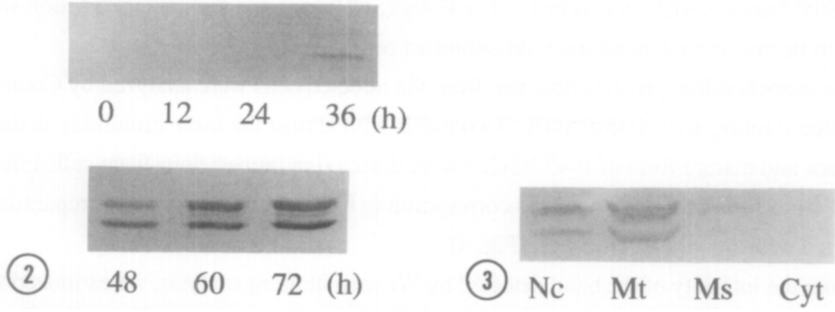

Fig. 2. Time course of P-450(SCC) expression in cells infected with AcNPV-scc(+). Infected cells were harvested at the indicated times (0, 12, 24, 36, 48, 60, 72 hours). Total cellular proteins (10 μg) were electrophoresed, and analyzed by Western blotting as described in Materials and Methods.

Fig. 3. Subcellular distribution of P-450(SCC) in infected cells. Four fractions were prepared from the homogenate of the infected cells. All the lanes are the Western blots of P-450(SCC) with 7 μg each of the four fractions (Nc, nuclei; Mt, mitochondria; Ms,microsomes; Cyt,cytosol).

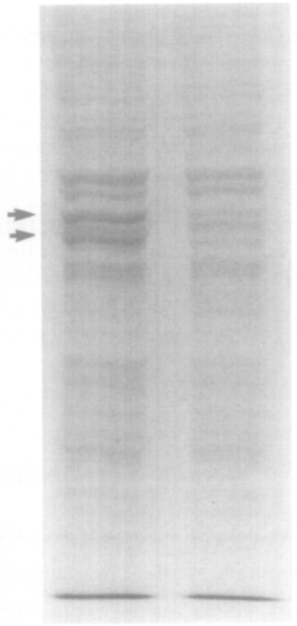

Fig. 4. SDS-PAGE of mitochondrial fractions from cells infected with AcNPV-scc(+), and -scc(-). Proteins(20 μg) of each mitochondrial fraction were separated by SDS-PAGE and stained by Coomassie Brilliant Blue. Arrows indicate the positions of the two bands on the preparation from the cells infected with AcNPV-scc(+) virus, having almost the same mobilities to those of the precursor and mature forms of P-450(SCC).

mitochondrial fraction was judged to be adequate by the facts that the specific activity of succinate-cytochrome c reductase (a mitochondrial marker) of the mitochondrial fraction was 46 times higher than that of the microsomal fraction, and that the microsomal fraction showed 12 times higher specific activity of NADPH-cytochrome c reductase (a microsomal marker) than the mitochondrial fraction. (data not shown) The P-450(SCC) found in the nuclear fraction was presumed to be due to contamination with unbroken cells.

The mitochondrial proteins obtained from the infected cells were analyzed by Coomassie Brilliant Blue staining after SDS-PAGE. Two bands with almost the same mobilities as those of the precursor and mature form of P-450(SCC) were detected in preparations from cells infected with AcNPV-scc(+) virus. However, no corresponding bands were observed in preparations from cells infected with AcNPV-scc(-) virus (Fig. 4).

From the intensity of the band detected by Western blotting analysis, we estimated the content of the mature form of P-450(SCC) to be 1.2μg per 1mg of mitochondrial protein, which is about one tenth of that in mitochondria from bovine adrenal cortex.

Most mitochondrial proteins are synthesized in the cytoplasm as larger precursor forms, and post-translationally imported into mitochondria where they are processed to their mature forms. The cytosol contains a pool of these precursor proteins, and their import into mitochondria is not strictly coupled to their syntheses [19]. For example, the F_1-ATPase β-subunit precursor

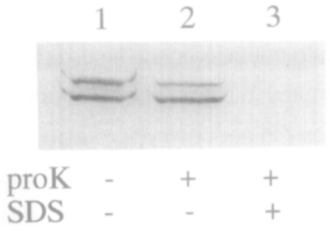

Fig. 5. Proteinase K treatment of P-450(SCC) in the mitochondrial fraction. Mitochondria were incubated at 30°C for 15 min in the absence (lane1) or presence (lane 2) of 20 μg/ml proteinase K. Digestion was also carried out in the presence of 0.5% SDS (lane 3). After the addition of PMSF, all the treated mitochondria were analyzed by SDS-PAGE, followed by Western blotting.

was purified to homogeneiety from its cytosolic pool in yeast cells [20]. In contrast, Fig.3 shows that the precursor form of P-450(SCC) was not detected in the cytosol, but found only in the mitochondria, suggesting its efficient import into mitochondria. Therefore, we analyzed the natures of both the precursor and mature form of P-450(SCC) found in the mitochondrial fraction.

As shown in Fig 5, the precursor of P-450(SCC) was partially resistant to externally added protease, while the mature form was fully resistant. In the presence of a detergent, both forms were completely sensitive to the protease. These data indicate that the protein that is resistant to protease is located within mitochondria, while a portion of the precursor that is sensitive to protease is located on their surface. The translocation of the precursor form across the mitochondrial membrane and its processing to its mature form are reported to be closely coupled [21, 22, 23]. However, Ou *et al.*[24] showed in a cell-free system that, unlike most other mitochondrial protein precursors, the translocation of P-450(SCC) precursor proceeds independently of its conversion to the mature form. Their conclusions are supported by our observation of the presence of the precursor form within mitochondria in intact insect cells.

Even though the expressed P-450(SCC) was transported into mitochondria and processed to the mature form, we could not demonstrate its activity in the mitochondria, by a sensitive method of HPLC or TLC, which could detect the production of as little as 20 pmol of pregnenolone. The CO-difference spectrum of the reduced form, a criterion to identify cytochrome P-450, was also not detectable. In the hope of increasing the amount and stability of functional P-450(SCC), we supplemented the culture medium before infection, with hemin chloride to enhance the heme synthetic capacity of the insect cells [25], and with 20-α-hydroxycholesterol, which is a substrate for P-450(SCC). However, these additions had no effect on the activity of the proteins expressed. Therefore, we concluded that most of the P-450(SCC) within mitochondria had little or no activity.

So far, no general strategy has been established for expressing functional heme proteins including cytochrome P-450 in a heterologous system. The conditions necessary for each protein seem to be different depending on the properties of these proteins. In this study, although the expression of P-450(SCC) at a high level and its efficient transport into mitochondria were achieved with the baculovirus expression system, the protein produced did not exhibit definite activity and seemed to exist as an inactive form. Therefore, the data reported here seem to indicate

that the imported and processed P-450(SCC) failed to adopt the correct conformation of the active enzyme, and/or that the protein could not incorporate heme effectively.

Recently, Wada *et al.* attempted to express both the precursor and mature form of P-450(SCC) in *Escherichia coli*. They did not observe production of the immunoreactive precursor form of P-450(SCC), but detected functional expression of the mature form of the enzyme. The recombinant P-450(SCC) expressed exhibited very similar enzymatic and spectral properties to those of the native protein [26]. Their bacterial expression system for the functional mature form of P-450(SCC) will be usefull in studying the reaction mechanism of this enzyme by site-directed mutagenesis.

Little is known about the process of folding of imported proteins into functional forms within mitochondria. There have, however, been various studies on how the precursors of mitochondrial proteins are recognized and imported into mitochondria [27]. Therefore, our system, in which large amounts of an inactive form of P-450(SCC) are accumulated, will be useful in elucidating the whole process of formation of active P-450(SCC).

Acknowledgments

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