Review

Reminiscence of our research on membrane phospholipids in mammalian cells by using the novel technology

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Abstract: By using "our devised up-to-the-second technique" over 30 years ago, we succeeded in the first isolation in the world of the three different kinds of mammalian cell mutants defective in the biosynthesis on each of phosphatidylserine (PS), cardiolipin (CL) and sphingomyelin (SM) from the parental CHO cells. As the results, we found that during the biosyntheses of PS and SM, the biosynthetic precursor or the final lipids are transported from their synthesized intracellular organelles to the plasma membranes via the other intracellular organelles. We further clarified the presence of the reversed routes for PS and SM from the plasma membranes to their synthesized organelles too. Our first epoch-making finding is not only the cycling interconversion reactions between PS and PE catalyzed by PSS-II and PSD but also their simultaneous transferring between MAM and Mit (found by O. Kuge). Our second finding is "the ceramide-trafficking protein (CERT)" working as the specific transfer protein of ceramide from the ER to the Golgi apparatus, during the SM biosynthesis (by K. Hanada).

As for their new biological roles, we clarified possible contribution of PS and/or PE to the fusion process between viral envelope and endosomal membrane, releasing the genetic information of the virus to the host cytoplasm. CL is contributing to the functional NADH-ubiquinone reductase activity by keeping the right structure of Coenzyme Q9 for its functioning. SM and cholesterol form the microdomain within the plasma membrane, so-called "the raft structure" where the GPI-anchored proteins are specifically located for their functioning.

Keywords: membrane phospholipids (PS, CL and SM), mammalian cell mutants, biosynthetic regulation, biological roles, lipid transport, intracellular organelles

1. Beginning

I started my scientific career for studying on phospholipids at the very early stage of their biology, when moving from Department of Tuberculosis, National Institute of Health (NIH) of Japan to Prof. Shoshichi Nojima's laboratory, supervised by Prof. Den-ichi Mizuno, Department of Pharmaceutical Sciences, the University of Tokyo, over 50 years ago. Since then I have been interested in the biological roles of membrane phospholipids, first in *Mycobacteria*,[1)–3)] and later in *Escherichia coli*,[4)] because the different genera of bacteria may have their own phospholipid species esterified with different fatty acyl groups. Accordingly my Doctor Thesis was focused on "Phospholipid Species, their Subcellular Distributions, and Metabolic Behaviors in *Mycobacteria*".

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Abbreviation: CHO: Chinese hamster ovary; PS: phosphatidylserine; PSA-3: phosphatidylserine auxotroph; PSS: phosphatidylserine synthase; PE: phosphatidylethanolamine; PC: phosphatidylcholine; PSD: phosphatidylserine decarboxylase; MAM: mitochondria-associated membranes; CL: cardiolipin; PG: phosphatidylglycerol; PGP: phosphatidylglycerophosphate; SPT: serine-palmitoyltransferase; GPI: glycosyl-phosphatidylinositol; LPS: lipopolysaccharide; SM: sphingomyelin; PI-PLC: phosphatidylinositol-specific phospholipase C; PLAP: human placental alkaline phosphatase; ER: endoplasmic reticulum; Mit: mitochondria; CERT: ceramide-trafficking protein.

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During my stay in Prof. John H. Law's laboratory, Department of Biochemistry, the University of Chicago for 3 years as a Fulbright exchange scholar, I clarified the molecular mechanism of tuberculostearic acid biosynthesis,[5)–7)] and further found the new methylation pathway for fatty acid methylester in *Mycobacteria*.[8)] Several years later, during my stay as a Visiting Professor in Rice University, Houston, I was promoted to the Director of Department of Chemistry, NIH of Japan.

2. My new start at NIH of Japan

As a Director, I decided to change my scientific work from bacterial cells to mammalian cells, because, in addition to my special interest in the biogeneses and biological roles of a wider variety of membrane phospholipids in mammalian cells, the host cell membranes could also play very important roles during the development of infectious diseases. Before starting our new project, we had to set up the necessary facilities, and further to get the essential knowledge for the mammalian cell culturing, and finally to devise the new techniques applicable to the updated lipid research. Then gradually the young scientists joined in my laboratory and were enthusiastically contributing to get the basic and the devised new techniques for the mammalian cell culturing and also the special knowledge on the fundamental characters of the strained mammalian cultured cells at the very early "so called tissue culture era" in Japan.

The metabolic pathways for major membrane phospholipids in mammalian cells were roughly understood.[9),10)] However the regulation on phospholipid metabolism in eukaryotes is not well understood except cholesterol[11),12)] and phosphatidylcholine[13)–15)] biosyntheses. Especially almost no information is available how membrane phospholipids biosynthesized in their specified organelles (mainly in the endoplasmic reticulum) are transferred to the other intracellular organelles.

Further, very interestingly there are several biosynthetic processes to convert from one phospholipid species to the others in mammalian cells. Among them we were interested in bio-conversion from phosphatidylethanolamine (PE) to phosphatidylcholine (PC) by the three successive trans-methylation reactions,[16)–20)] and also in inter-conversions among PC, PS (phosphatidylserine) and PE by the base-exchange reactions, because these biosynthetic reactions within the mammalian cells could alter the physico-chemical character of the bio-membranes, possibly leading to bio-transformation of the membrane functions. (The chemical structures of these phospholipids are shown in Fig. 1). The former project was carried out mainly by the young, newcomer scientists in my laboratory as our first trials to get the basic character of the various strained mammalian cultured cell lines,[21)–22)] and the results of the latter project are described in the following PS section of this review.

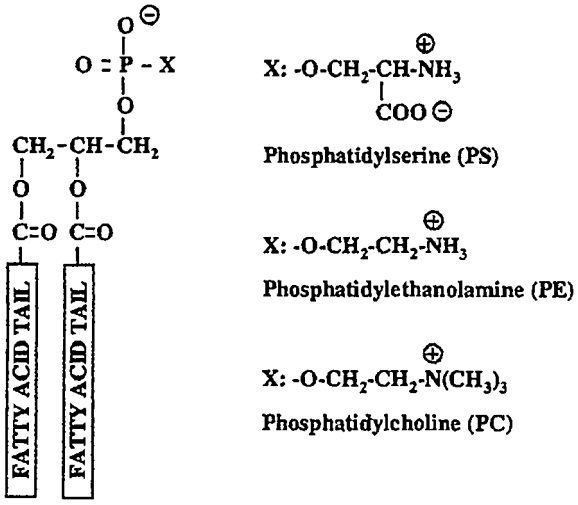

Fig. 1. Structures of phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine.

Isolation and biochemical characterization of the several mammalian cell mutants with specific defects in the biosynthesis of the specified phospholipid are a powerful approach to clarify its metabolic regulations and also its biological roles.

In this review, I briefly summarized the major achievement of our research clarified by using the three different kinds of biosynthetic mutants defective in each of phosphatidylserine (PS), cardiolipin (CL) and sphingomyelin (SM).

3. Phosphatidylserine (PS); This is our first project

Phosphatidylserine (PS) is one of the major phospholipids in mammalian cells, and occupies about 10% of the total membrane phospholipids. In bacteria and yeast, PS is synthesized through the reaction of L-serine with CDP-diacylglycerol catalyzed by PS synthase. Mammalian cells contain a base-exchange enzyme which catalyzes the exchange of the polar head groups of pre-existing phospholipids with free serine, choline and ethanolamine to produce PS, PC and PE[23)] (Fig. 1). However the physiological

roles of the enzyme are unknown, and the molecular mechanism of PS biosynthesis in mammalian cells was not yet well understood.

As for the biological roles of PS when we started our project, we only knew that PS is involved in the blood coagulation,[24] and also is the cofactor of protein kinase C in the receptor-mediated signal-transduction.[25]

3-1. Isolation of somatic cell mutants defective in phosphatidylserine (PS) biosynthesis. We (M. Nishijima, O. Kuge, and the coworkers) succeeded to isolate the two kinds of such mutant clones from the parental CHO-K1 cells.

3-1-1. A CHO-K1 cell mutant defective in PS biosynthesis with the thermo-labile activity of the base-exchange reaction with choline. We first tried to get the thermo-labile mutant of PS-biosynthesis, because PS could be essential for cell growth. So our first CHO cell mutant clone (designated as mutant 64-ts) with thermo-labile defect in the base-exchange reaction of phospholipids with choline was isolated from the parental CHO-K1 cells by using an *in situ* enzymatic assay for the reaction in cell colonies immobilized on polyester cloth[26] (Fig. 2). The specific activity of the choline-exchange reaction in extracts of mutant 64-ts was 6% of that of the parent CHO cells at the non-permissive temperature (40 °C). The choline-exchange activity in mutant 64-ts was more thermo-labile in cell extracts than that in CHO cells, suggesting that a mutation is induced in the structural gene for the choline-exchange enzyme. Labeling of the intact cells with ^{32}P clearly showed that mutant 64-ts cells was defective in the biosynthesis of PS, but not in PC biosynthesis at 40 °C. However, both the serine-exchange and the ethanolamine-exchange activities in mutant 64-ts *in vitro* decreased only to the half of those of CHO cells.

Growth of mutant 64-ts cells continued only for two divisions at 40 °C. However we found that the exogenous addition of PS to the culture medium restores the growth rate of mutant 64-ts to the CHO level.[26),27] Further the temperature-resistant revertant isolated from mutant 64-ts exhibited nearly normal ability to synthesize PS at 40 °C and also showed the choline-exchange activity *in vitro* similar to that in CHO cells.

All these our findings described above confirmed of our preliminary work that the choline-exchange enzyme works as the major route for the biosynthesis of PS *in vivo* and that the temperature sensitive growth of the mutant 64-ts comes from a defect in PS biosynthesis at 40 °C.[26]

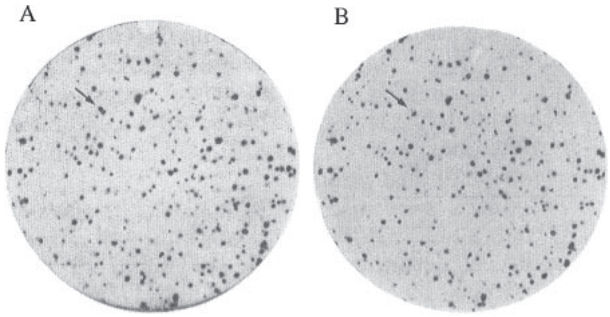

Fig. 2. Identification of CHO cell colony defective in the choline-exchange reaction. (A) Coomassie blue-stained polyester disc. (B) Autoradiogram of the polyester disc. The arrows mark the position of the mutant colony. Two polyester cloths with cell colonies (A and B) are replicated from the master plate.

3-1-2. Another CHO-K1 cell mutant, PSA-3, (PS-Auxotroph; the PS requiring mutant for growth).[28] In order to confirm the above described character of mutant 64-ts which requires exogenously added PS for growth at the non-permissive temperature, we also isolated a CHO cell mutant requiring PS for growth, PSA-3 (a PS auxotroph) as our second mutant possibly defective in PS biosynthesis *in vivo* by using the replica technique with polyester cloth. Mutant PSA-3 was found with almost the same character of our first mutant 64-ts except thermo-lability.[27),28] So the further work was mainly carried out with this auxotroph.[28]

3-2. Characterization of mutant PS-Auxotroph (PSA-3).

3-2-1. Presence of two kinds of serine-exchange enzymes.[27),28] As expected, our new mutant, PSA-3 requires exogenously added PS for cell growth. The *in vivo* labeling experiments with ^{32}P and L-[U-^{14}C]serine revealed that mutant PSA-3 was strikingly defective in PS biosynthesis. After PAS-3 cells were cultured for 2 days without exogenous PS supply, their PS and PE contents decreased to one-third and an half of those of the parent, respectively.

The base-exchange activities of PSA-3 *in vitro* with choline, ethanolamine and serine were 1, 45 and 33% of those of the parental CHO-K1 cells respectively. Furthermore, as shown in Fig. 3, the serine-exchange activity of CHO cells *in vitro* was decreased approximately up to 40% by addition of excess choline in the reaction mixture while that of PSA-3 was not significantly affected. On the contrary, excess ethanolamine effectively inhibited serine-exchange activities of both CHO and PSA-3.

The results described above led us to the new findings, which we concluded as follows;

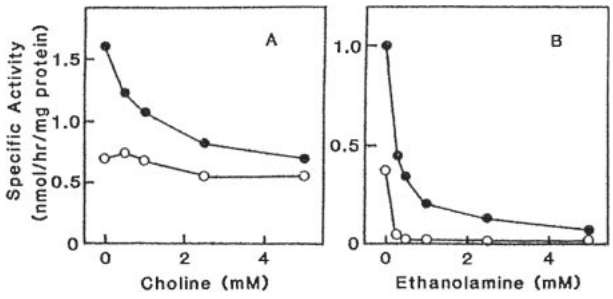

Fig. 3. Inhibition of the serine-exchange activity by choline (A) and ethanolamine (B). The serine-exchange activity in cell extracts prepared from CHO-K1 (●) and mutant PSA-3 (○).

(a) There are at least two kinds of serine-exchange enzyme (hereafter we named as phosphatidylserine (PS) synthase, abbreviated as PSS) in CHO cells. Serine-exchange enzyme I (PSS-I) can catalyze the base-exchange reactions of phospholipids with serine, choline and ethanolamine, whereas serine-exchange enzyme II (PSS-II) can use both serine, and ethanolamine as substrates but not choline.

(b) Serine-exchange enzyme I (PSS-I), which is defective in PSA-3, plays a major role in phosphatidylserine biosynthesis in CHO cells. Thus CHO cells have both PSS-I and PSS-II for the biosynthesis of PS while mutant PAS-3 cells have only PSS-II but not PSS-I.

(c) Serine-exchange enzyme I (PSS-I) is essential for the growth of CHO cells.

3-2-2. Utilization of PC and PE as substrates for PS biosynthesis in vivo. We clarified the substrate phospholipids for the biosynthesis of PS by the base-exchange reactions.[29] By culturing the parent CHO cells and the mutant PSA-3 cells in the presence of [^{32}P]-PC, only CHO cells, but not PSA-3 cells, accumulated [^{32}P]-PS, indicating that PC is the substrate of PSS-1 *in vivo*. On the contrary in the case of [^{32}P]-PE instead of [^{32}P]-PC, both PSA-3 cells and CHO cells accumulated [^{32}P]-PS, but PSA-3 cells accumulated the radioactivity in PS more efficiently than CHO cells, indicating that PE is the substrate of PSS-II *in vivo*.[29] We further clarified that exogenously added PC is utilized for PS biosynthesis in CHO cells, but not in PSA-3 cells. These findings are an additional proof for the presence of both PSS-I and II in CHO cells but only PSS-II in PSA-3 cells.

Furthermore we found that mutant PSA-3 cells grew normally in the media supplemented with either PE or PS, and that the biosynthesis of PS in PSA-3 was normal in the presence of exogenous PE in the medium. The simplest explanation of these findings is that PS in CHO cells is biosynthesized through the following sequential reactions: PC→PS→PE→PS. These three successive reactions are catalyzed by PSS-I, phosphatidylserine decarboxylase (PSD) and PSS-II, respectively.[29]

The reason why mutant PSA-3 with the normal activity of PSS-II could not synthesize the essential amount of PS *in vivo*, was also proved as an insufficient supply of either the substrate PS for PSD-enzyme or the substrate PE for PSS-II enzyme, because PS is mainly supplied in CHO cells by PSS-I from PC, and subsequently metabolized to PE by PSD.[29]

3-2-3. Intracellular transferring of PS and PE during the three successive chain reactions. "Both PSS-I and -II were initially understood in the endoplasmic reticulum (ER), but recently they are mainly located in the mitochondria-associated membranes (MAM),[30)–32)] but still we cannot exclude their possibility in the ER. However PSD is present in the inner membrane of mitochondria (Mit).[33]

Judging from the results of the three successive reactions catalyzed by each of the three enzymes described above and also in Fig. 4, PS, PE and again PS are sequentially synthesized as the reaction products, with the simultaneous intracellular-transferring of PS from mainly MAM to mitochondria, and also of PE from mitochondria to mainly MAM.

These cycling routes of PS and PE between mainly MAM and mitochondria are especially interesting and important from the view point of not only the phospholipid sorting from their newly synthesized intracellular organelles to their finally destined plasma membrane via the other intracellular organelles, but also phospholipid transferring through the reversed routes from the out side of the cells. Because MAM is a main supplier of PS within the cells and so PS has to be transferred from MAM to mitochondria and further simultaneously or separately to the other intracellular organelles by using the right sorting route of phospholipids. On the contrary, mitochondria (together with the ER) is the main supplier of PE, and so PE also has to be transferred from mitochondria not only to MAM and the ER but also to the other intracellular organelles as well by using the right sorting routes of phospholipids. These intracellular transferrings not only of PS and PE, but also of the other lipids by utilizing the right, sorting route and/or the reversed route for phospholipids are the new finding, which could clarify the biogeneses of biological membranes

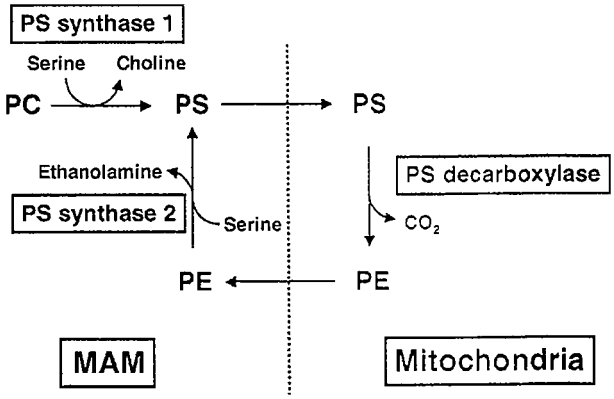

Fig. 4. PS decarboxylation pathway in mammalian cells. PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine. MAM, mitochondria associated membranes.

in detail. We also found "the similar inter-organelles trafficking system for ceramide in the following sphingomyelin section of this review."

3-3. Genetic evidence from the two complementary cDNAs encoding PSS-I and PSS-II, and further the suppresser cDNA encording PSD which could also restore the growth of PSA-3 cells. Unexpectedly we (O. Kuge, K. Saito, M. Nishijima, and the coworkers) isolated the three different kinds of cDNAs. Among them the two cDNAs encording PSS-1 and PSD could complement the PS-requirement of PSA-3 cells.

3-3-1. The first cDNA (designated as *pssA*) was proved as the cDNA encoding PSS-I by its complementation of the PSS-I in PSA-3 cells, as expected. Namely the serine, ethanolamine and choline base-exchange activities in the transformant cells are 6.2-, 6.7- and 15.6-fold those of the parent CHO-K1 cells, respectively.[31),34)]

3-3-2. As for the cDNA encoding PSS-II, O. Kuge, and his coworkers got the information of "a partial human cDNA encording a PSS-I related peptide" in the DNA Data Base. By utilizing this human cDNA information, finally he cloned a CHO cDNA designated as *pssB*, which encode a protein with 32% amino acid sequence identity with CHO PSS-I. The *pssB* is proved to encode PSS-II from the evidence that its expression in the parent CHO cells resulted in big increase of both serine- and ethanolamine-exchange activities but not choline-exchange activity at all.[35)]

Expression of *pssA* even in the insect cells increased all the serine-, ethanolamine- and choline-base-exchange activities, while that of *pssB* elevated only serine- and ethanolamine-base-exchange activities. These findings clearly indicate that the *pssA* encodes PSS I, and the *pssB* does PSS II. This is a genetic proof for the presence of two kinds of PSS in CHO cells.

3-3-3. We also cloned the third gene from the genomic fragments of the parent CHO-K1 cells, designated as *pssC* gene which also complements the PS requirement of PSA-3 cells for growth.[36)] By computer-search through a protein data bank, we found that *pssC* gene had homology with E. coli *psd* encoding phosphatidylserine decarboxylase (PSD) at the amino acid level. Introduction of the cloned *pssC* gene into the mutant PSA-3 cells resulted in a 2-fold increace in the PSD activity.[36)] The increased activity of PSD is further confirmed by introducing *pssC* gene into yeast. The cDNA encoding PSD was later isolated by using the genomic fragment of *pssC* gene as a probe.[36)]

The reason why *pssC* gene complements the defect of PSA-3 is 1) loosening up the rate-limiting step in the PS-PE cycling catalyzed by additional supply of PSD within the cells, and 2) consequently enough supply of PS and PE by utilization of the additional PE synthesized through the different, "so-called Kennedy pathway" in the ER, and 3) possibly further supply of PS and PE not only from mitochondria and MAM (and the ER), but also from the other intracellular organelles via their reversed supply route, especially in PSA-3 cells.

The *pssC* gene is a suppressor gene encoding PSD. This finding suggests that PSD plays an important role in the formation of both PS and PE.[36)]

3-4. Regulation of PS biosynthesis. As for the PS biosynthesis, the final products are not PS in MAM but all the PS present in the intracellular organelles including plasma membranes, because PS just synthesized in MAM has to be transferred through the right sorting route of PS for further contribution to the membrane biogeneses of the intracellular organelles including plasma membranes. However, as described already, we found that PS-biosynthesis in CHO-K1 cells is remarkably inhibited by the addition of PS to the culture medium[27)] by transferring of PS through the reversed route from the plasma membranes to MAM. This inhibition of PS biosynthesis by PS could be explained by the presence of "the new type of feed back inhibition or control" involved in the regulation of PS biosynthesis. It was later proved by us and also internationally approved, since we found that both the right sorting route and also the reversed one for the PS-transferring are working from MAM to the plasma

membranes via various intracellular organelles and also visa versa within the cells. So at present the PS-transferring system is also included in the "feed back control or inhibition" in the lipid field during the membrane biogenesis.

We also found that PS directly inhibits the PS synthase activity in isolated membrane fractions of CHO-K1 cells, indicating that the product inhibition in appearance occurs on PS synthase activity *in vitro*.

We have fortunately isolated a CHO cell mutant, named as 29, defective in the "feed back control or inhibition of PS biosynthesis by PS".[37] By addition of PS to the culture media, PS biosynthesis in CHO-K1 cells is reduced by 98%, while that in mutant 29 cells is only by 29%. Furthermore without PS in the culture media, mutant 29 synthesizes PS at a 2–3 fold higher rate and exhibits an approximately 2 fold higher PS contents than those of CHO-K1 cells. In an *in vitro* assay system, the PS synthase activities of CHO-K1 and mutant 29 cells are essentially the same. However, the PS synthase activity of CHO-K1 cells is inhibited by exogenously added PS, but that of the mutant cells is not.[37] Later our group clearly proved that mutant 29 has a point mutation in the PSS-I gene which results in the replacement of Arg-95 in PSS-I with Lys. The introduction of the R95K mutated PSS-I cDNA into CHO-K1 cells induces about a 5-fold elevation of the PS biosynthetic rate and about 2-fold elevation of the cellular PS level without exogenous PS. CHO PSS-I and -II are similar in sequence each other with 38% identity of their amino acid sequences between the two synthases. The Arg-97 is also observed in PSS-II gene in the overproducer of PSS-II and proved to be critical for depression of the overproduced PSS-II activity.[37] All these results were obtained by analyses of the mutant 29 from the molecular level.

However, "contribution of all the PS including the exogenously added and also present in various intracellular organelles to the feed-back control or inhibition on PS biosynthesis" has already clarified and established in CHO cells.

3-5. Biological roles of PS and/or PE during an enveloped virus infection and maturation. As schematically shown in Fig. 5, mammalian cell membranes play important roles during the enveloped virus infection and maturation, which include internalization of virion in coated vesicles via adsorptive endocytosis, fusion of the viral membrane with endosomes, whereby the nucleocapsids released into the cytosol, and finally biosynthesis and assembly of the virus. Among them we were interested in the functions of cellular phospholipids during the interactions between viral envelope and the host cell membranes, because only a few reports including ours[22] have focused from this point of view.

As described in a preceding section, mutant PSA-3 cells have almost no PSS-I enzyme, and so when they were cultured for 36 hours without exogenous PS in the culture medium, their PS and PE contents decreased up to 40% and 60% of their original ones, respectively. Sindbis virus production in the mutant cells decreased immediately after PS deprivation, and 24 hours after starvation of PS, it came down to the one hundredth of the original colony forming unit **(cfu)** of the virus (Fig. 6).[38] However the cell growth, viability and syntheses of protein, DNA and RNA of PSA-3 cells remain normal for approximately 40 hours after PS starvation. So the binding and internalization of the virus could occur normally, but the yield of virions and viral RNA synthesis were greatly reduced in PSA-3 cells under starvation of PS, suggesting that viral nucleocapsids are not normally released into the cytoplasm.[38]

Recently, several mammalian cell mutants resistant to enveloped RNA viruses were shown to be defective in acidification of endosomes, which, together with other findings, provides strong evidence that acidification of endosomes is required for the release of viral nucleocapsids from endosomes with the normal membrane phospholipids into the cytoplasm.[39] Judging from the sensitivity of PSA-3 cells to the diphtheria toxin,[38] the adsorptive endocytosis of diphtheria toxin,[38),39] including acidification of endosomes, is normal even in PSA-3 cells grown without PS, leaving the possibility that the endosomal membrane with the decreased content of PS and/or PE lowers the membrane fusion between viral envelope and the endosomal membrane even under the acidic circumstance.[38]

We next examined whether penetration of the internalized virus into the cytoplasm was normal or not by measuring the synthesis of Sindbis virus RNA, and got the dramatical results that the viral RNA synthesis greatly decreased only in the PSA cells grown without PS for 24 hours, compared with in the parent cells.[38] And further we also confirmed that the production of viral RNA in PSA-3 cells grown in the presence of PS was similar to that in CHO-K1 cells grown in both its absence and presence.[38] These results, as expected, clearly indicate the inefficient fusion of the envelope of Sindbis virus not only with the plasma membrane deficient

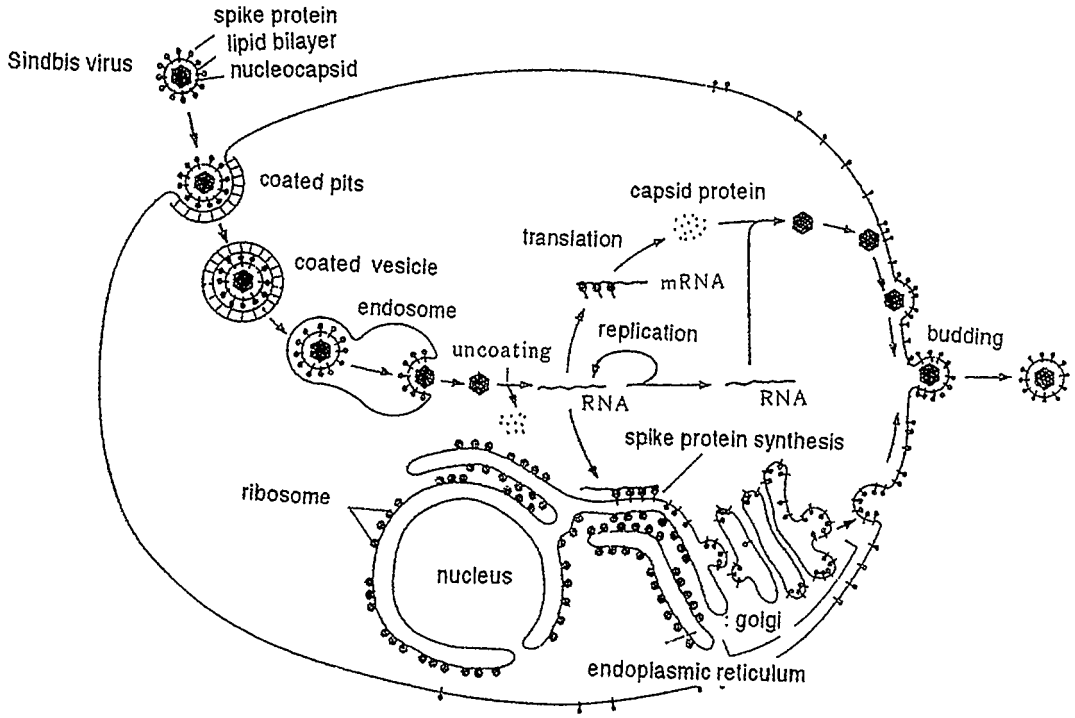

Fig. 5. Schematic demonstration of Sindbis virus infection and its maturation.

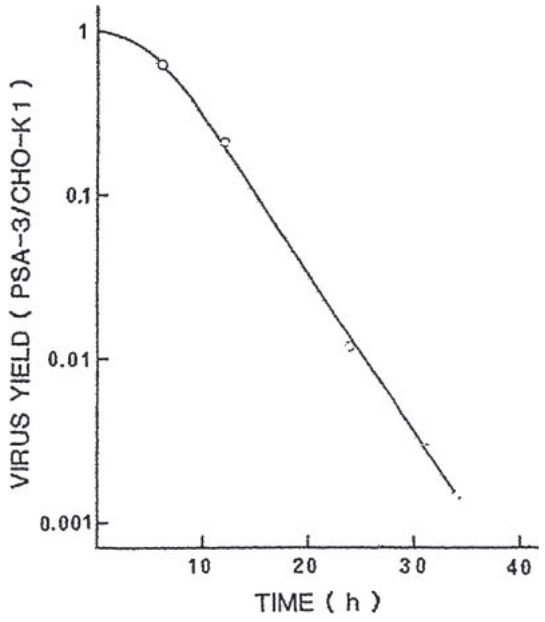

Fig. 6. Effect of phosphatidylserine starvation on Sindbis virus production in CHO-K1 and PSA-3 cells. Data are expressed as the ratio of the virus yield in PSA-3 to that in CHO-K1 cells.

in PS and/or PE, but also with the endosomal membranes deficient in PS and/or PE in PSA-3 mutant under PS-starvation.[38]

From these results together with the others, we first speculated and finally concluded that the cellular PS and/or PE in CHO-K1 contribute to the fusion step of the envelope of Sindbis virus with the endosomal membrane, while the PS and/or PE contents in PSA-3 cells under PS starvation are not sufficient for contributing to such a fusion process.[38]

4. Cardiolipin (CL) (and Phosphatidylglycerol (PG)). Our second project

Both cardiolipin (CL) and phosphatidylglycerol (PG) are important constituents of membrane phospholipids not only in animals, but also in plants and microorganisms. The biosynthetic pathway of PG and CL in mammalian cells was suggested by the early enzymological studies.[40),41)] Both phosphatidylglycerophosphate (PGP) synthase and PGP phosphatase contribute to the biosynthesis of PG, and further cardiolipin (CL) synthase catalyzes the transfer of phosphatidic acid from CDP-diglyceride to phosphatidylglycerol (PG) (Fig. 7).

We had scientific interest in the biological role of CL, because it is primarily present in mitochondria, almost exclusively in their inner membranes, in most mammalian cells, and constitutes up to 20% of the total phospholipids in mitochondria.[41)] Thus CL was expected to contribute to expression of the mitochon-

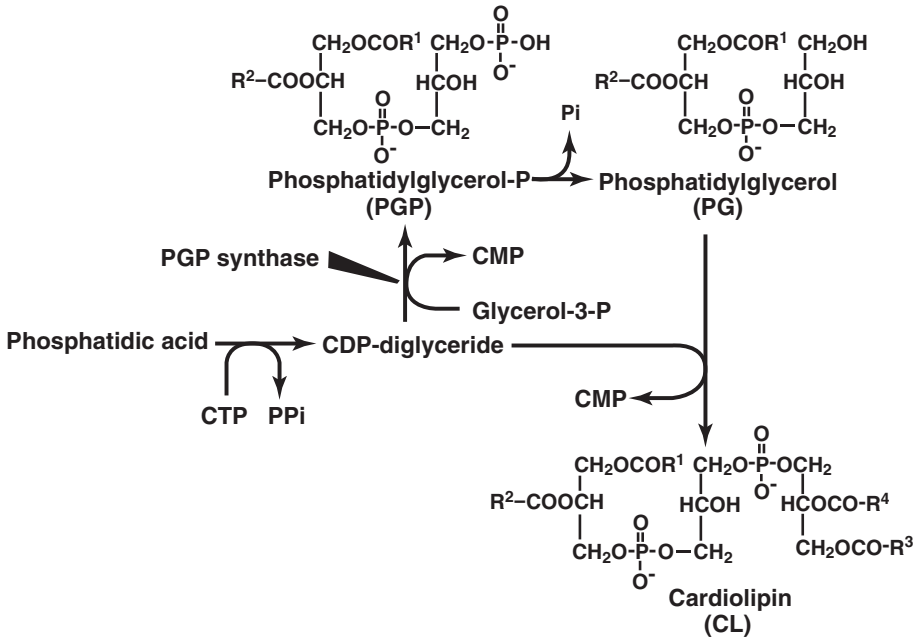

Fig. 7. Biosynthetic pathway for PG and CL. R^1, R^2, R^3 and R^4 represent hydrocarbon chains of fatty acid residues.

drial functions, but no conclusive role of CL *in vivo* has ever been found.[41] It is our main purpose to clarify the biological role of CL in mitochondria *in vivo*.

On the contrary, PG is a rather minor constituent, only less than a few % of the total phospholipids, in mitochondria of mammalian cells, and it is not specifically localized among the intracellular organelles including plasma membrane. However PG content is known up to 10% of the total phospholipids in the lamellar body of the lung where PG works as pulmonary surfactant.[42),43)]

Both CL synthase[44)] and also PGP synthase[45)] were recently purified from mitochondrial membranes, and especially CL synthase is exclusively located in mitochondrial inner membranes, indicating that these two phospholipids were originally synthesized in mitochondria, and so they have to move from mitochondria to the other organelles including the plasma membrane. This is also the very interesting issue to be proved.

4-1. A CHO cell mutant specifically defective in PGP synthase, (with impaired biosyntheses of both phosphatidylglycerol (PG) and cardiolipin (CL).[46)] Phosphatidylglycerophosphate (PGP) synthase is the key enzyme for biosyntheses of polyglycerophospholipids, namely both PG and CL (Fig. 7). We (T. Ohtsuka, O. Kuge, M. Nishijima, and the coworkers) have successfully isolated the CHO cell mutant with a thermo-labile defect in the PGP synthase activity (designated as PGS-S) by using an *in situ* enzyme assay.[46)] The PGP synthase activities in cell extracts of mutant PGS-S grown at the permissive (33 °C) and the non-permissive temperatures (40 °C) were 14 and 1% of those in the parent CHO cells, respectively. Mutant PGS-S also showed thermo-labile defect in biosyntheses of PG and CL *in vivo*, leading to decreace of both PG and CL contents, together with thermo-lability of cell growth. A temperature-resistant revertant (R6) isolated from mutant PGS-S simultaneously restored the PGP synthase activity and also the ability to synthesize PG and CL *in vivo*, all these restorations clearly indicating genetic evidence that PGP synthase is responsible for both PG and CL syntheses, and is further essential for cell growth.[46)] This mutant PGS-S is the first mammalian cell variant with a specific lesion in PGP synthase.

4-2. Mitochondrial disfunction of mutant PGS-S deficient in cardiolipin (CL).[47)] In order to clarify the biological role of CL, first of all, we compared the structure and function of mitochondria in mutant PGS-S cells grown at 40 °C with those of CHO cells, because, as expected, the CL synthesis was impaired in mutant PGS-S. And we found that the mutant showed both morphological and functional abnormalities in mitochondria. As for the morphological change, all the mitochondria in

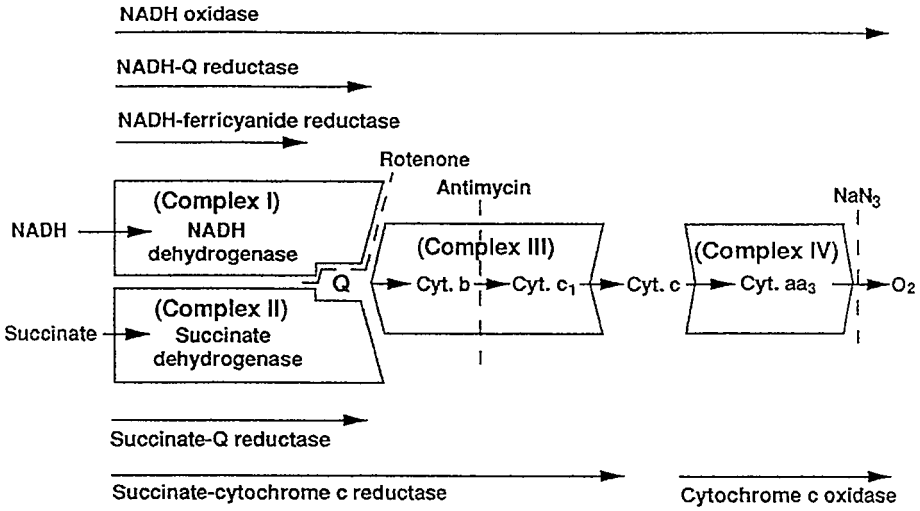

Fig. 8. A scheme of electron transport chain. The dotted lines indicate possible inhibition sites by the respective inhibitors. Cyt, cytochrome.

Table 1. The functional changes in the electron transport chain in mitochondria under starvation of CL

Strain	Temperature	Oxygen consumption[a]		NADH oxidase[b]		NADH-Q reductase[b]			
						−DBH		+DBH (50 μM)	
	°C	*nmol/min/10⁷ cells*	%	*nmol/min/mg*	%	*nmol/min/mg*	%	*nmol/min/mg*	%
CHO-K1	33	8.0 ± 0.4	100	24.0 ± 3.2	100	6.7 ± 0.7	100	28.9 ± 6.2	100
PGS-S	33	10.1 ± 0.5	126	16.7 ± 1.2	70	6.1 ± 1.3	91	37.9 ± 1.6	131
CHO-K1	40	6.5 ± 0.1	100	16.5 ± 2.4	100	6.2 ± 1.7	100	35.4 ± 2.8	100
PGS-S	40	4.1 ± 0.1	63	6.8 ± 1.8	41	1.3 ± 1.1	21	32.5 ± 4.2	92
R6	40	5.5 ± 0.2	85	14.6 ± 0.2	88	4.5 ± 0.2	73	ND[c]	

a) Oxygen consumptions are measured by the intact cells. b) NADH oxidase and NADH-ubiquinone reductase activities are assayed by using the cell extracts of CHO-K1, PGS-S and R6 cells. c) Not determined.

mutant PGS-S appeared greatly enlarged and swollen, and further cristae were lacking or disorganized.[47] All these abnormalities were more clearly found by more stringent temperature sensitivity for cell growth in glucose-deficient medium and by reduced ATP production, increased glycolysis, and reduced oxygen consumption in intact cells.[47]

As for the functional change, by using the cell extracts of mutant PGS-S cells cultured at 40 °C, we found that the activity of rotenone-sensitive NADH oxidase was greatly reduced in mutant PGS-S (Fig. 8, Table 1). In the absence of exogenously added ubiquinone analogue, the activity of NADH-ubiquinone reductase (Complex I) was also greatly reduced in the mutant under the CL content reduced to about 20% of that of the parent CHO cells, whereas its activity was completely restored by the addition of exogenous DBH (2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone), a synthetic analogue of ubiquinone-2 with more hydrophilic character than the native ubiquinone 9 (Table 1). These results clearly indicated that the normal level of NADH dehydrogenase is present in the mutant. The reduced Complex I activity of the mutant cells in the absence of exogenous ubiquinone appears not to be due to a low content of the endogenous ubiquinone 9, since we found that the endogenous ubiquinone 9 contents in both the mutant and the parental CHO cells were nearly identical. Furthermore, both succinate-ubiquinone reductase (Complex II) activity assayed without addition of exogenous ubiquinone and succinate-cytochrome C reductase (Complex II and III) (Fig. 8), both of which are dependent on endogenous ubiquinone, were not significantly affected in the mutant. These results exclude the possibility that the functional concentration of ubiquinone is reduced

in the mutant. The other activity of the electron transport chain such as cytochrome c oxidase (Complex IV) was not significantly modified (Fig. 8). These results together with others indicated that the deficiency in mitochondrial respiration in mutant PGS-S cells was due to a prominent deficiency in Complex I activity, leading to the conclusion that CL is contributing to maintain the functional structure only for NADH–ubiquinone reductase, and possibly for natural ubiqinone-9, too (Complex I), but not for succinate-ubiquinone reductase (Complex II) as a whole. The different characters for the requirement of CL between Complexes I and II in the respiratory chains of the mutant could be explained by their different sensitivities of CL requirement for the expression of their enzyme activities; namely, for their different binding abilities and/or Km values to CL between Complexes I and II.[47)]

These results suggest that CL plays a critical role in mitochondrial functions, at least in the respiratory electron transport chain.

CL is required not only for normal respiratory chain reactions but also for other mitochondrial or cellular functions, because just compared with the other respiration mutants isolated so far,[48)] our PGS-S mutant could not grow even in the presence of glucose under the non-permissive temperature.

Since no mutant defective in biosynthesis of CL had been identified among the respiration-deficient strains, our PGS-S strain is the first such mutant to be recorded.

4-3. Isolation of cDNA encoding PGP synthase. Later K. Kawasaki and his coworkers[49)] isolated a CHO cDNA encoding a putative protein similar in sequence to the yeast PGS1 gene product, PGP synthase. By transfecting the isolated CHO cDNA (named as PGS1) to the mutant PGS-S, the cDNA-tranfected mutant PGS-S exhibited not only 620-fold and 7-fold higher PGP synthase activity than the non-transfected mutant PGS-S and the parental CHO K1 cells, respectively, but also restored the cellular CL and also PG contents, leading to simultaneous disappearance of its morphological and functional abnormalities[49)] in mitochondria. These results clearly indicate that the morphological and functional defects displayed by the mutant PGS-S are due directly to the reduced ability to make normal levels of CL (and/or. PG).

It should be feasible to isolate additional respiration-deficient mutants with a lesion in CL biosynthesis, possessing different genotypes from mutant PGS-S. Furthermore, similar metabolic disturbances affecting mitochondrial respiration and energy production in mammalian cell mutants have been observed in human metabolic diseases with defects in respiratory chain complexes and in syndromes with mitochondrial encephalomyelopathy.[50)] Thus, mutant PGS-S will be useful to increase our understanding of human mitochondrial diseases and will also be very valuable in elucidating the physiological importance of CL in mammalian cells.

On the contrary, PG is just present in very small amount even in mitochondria. However PG has the important role in the biosynthesis of CL as an immediate precursor within the cell. And further its content is known up to 10% of the total phospholipids in the lamellar body of the lung where PG works as a pulmonary surfactant.[42),43)]

5. Sphingolipids, with the special focus on sphingomyelin (SM). Our third project

Sphingolipids are ubiquitous constituents of the biological membranes in mammalian cells,[51)] and defined as lipids containing sphingoid bases (1,3-dihydroxy-2-amino-alkane and its derivatives) as a structural backbone.

A phosphosphingolipid, sphingomyelin (SM), typically amounts to 2–15% of the total phospholipids of mammalian cells, being the most abundant sphingolipid in almost all mammalian cells, and its subcellular localization is mainly in the plasma membranes.[52)] In a proposed pathway for sphingolipid biosynthesis, serine palmitoyltransferase (SPT) catalyzes the first step reaction, in which L-serine condenses with palmitoyl-CoA to produce 3-ketodihydrosphingosine (3-KDS). 3-KDS is enzymatically converted to dihydrosphingosine, subsequently to sphingosine, and then to ceramide. It is further converted to sphingomyelin (SM) and also glycosphingolipids, as shown in Fig. 9. However the detailed molecular mechanism for the total biosynthesis of SM was still controversial when we started our project. So we tried to clarify the biosynthetic regulations as well as the biological roles of SM, first of all, by isolating the mutants defective in sphingolipid biosynthesis.

5-1. The mutant (SPB-1) with thermo-labile serine-palmitoyl transferase (SPT).[52),53)] We (K. Hanada, M. Nishijima and the coworkers) devised an *in situ* screening system for the SPT activity and succeeded to isolate the temperature-sensitive mutant (SPB-1) with thermo-labile activity of serine-palmitoyl transferase (SPT) from the parental CHO

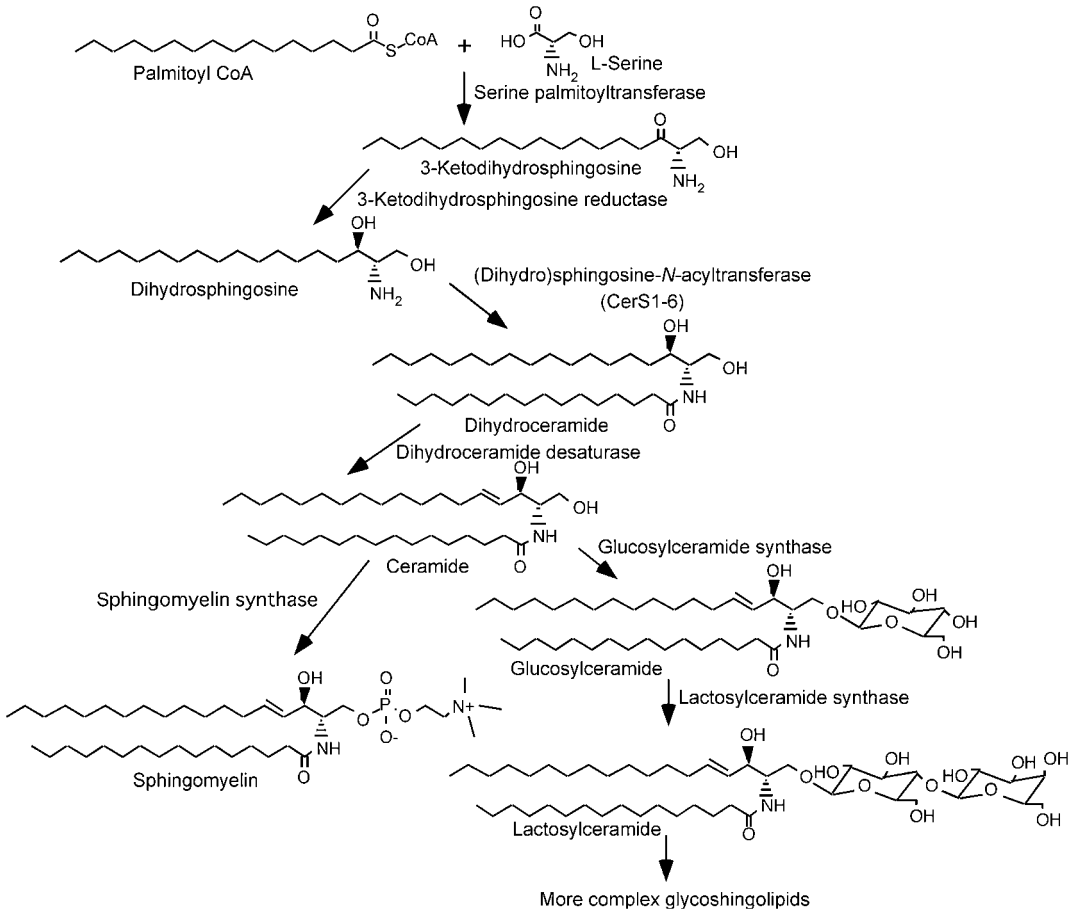

Fig. 9. Biosynthetic pathway of sphingolipids in mammalian cells.

cells.[53] The mutant SPB-1 cells stopped growth at 40 °C (the non-permissive temperature) after several generations, but at 33 °C (the permissive temperature) they have the similar growth rate to that of the parent CHO cells. The SPT activity in cell homogenates of mutant SPB-1 was, as expected, decreased to the negligible level at 40 °C.

The rates of the de novo syntheses of sphingolipids in mutant SPB-1 were much slower at 40 °C than at 33 °C, in contrast to those in the parent CHO cells. The SM content in the mutant cultured at 40 °C for several generations was also much less than that at 33 °C. These results, together with the others, clearly indicate that SPT functions in the main pathway for sphingolipid biosynthesis, and also sphingolipid(s) plays an essential role for cell growth.[53]

Very interestingly, we further found that the exogenously-added sphingosine in the culture medium restored the contents of both SM, and GM3 (ganglioside sialyl lactosylceramide as a representative of glycosphingolipid) in mutant SPB-1 cells near to the parent levels through its metabolic utilization and allowed the mutant cells to grow even at the non-permissive temperature. Similarly exogenously-added SM also restored the SM levels but only partly the GM3 levels, further suppressing the temperature-sensitivity for growth of mutant SPB-1 cells. In contrast, exogenously added glucosylceramide, which restored the GM3 levels but not the SM levels, failed to suppress the temperature sensitivity for growth of mutant SPB-1 cells. From these results we clarified that the temperature sensitivity for growth of mutant SPB-1 cells was due to the lack of cellular SM.[54]

5-2. Approach to the biological role of sphingomyelin (SM). Several biological roles of sphingolipids have already been suggested. First of all, sphingoid bases, N,N-dimethylsphingosine, lysosphingolipids and ceramide participate in the regu-

lation of protein kinase C and other kinase activities as physiological repressors in mammalian cells both *in vitro* and *in vivo*.[55)–58)]

Furthermore, phosphorylsphingosine may mediate intracellular calcium release through a C-kinase-independent pathway.[59),60)] So the metabolic turnover of SM is argued to be involved in the regulation of signal transduction.[61),62)]

SM itself in membranes could be related to the fluidity, osmotic fragility, and permeability,[63)] and may influence the transport or metabolism of cholesterol in mammalian cells because depletion of cellular SM alters intracellular distribution of cholesterol,[64)] and further, the mutant cells deficient in lysosomal sphingomyelinase accumulate not only SM but also cholesterol abnormally.[65)]

Apart from evidence on the biological roles of SM described above, we tried to find out the new roles of SM in the membranes by using mutant SPB-1 cells.

5-2-1. SM protects GPI-anchored protein.[66)] Both SM and glycosphingolipids are mainly located in the plasma membrane and especially enriched in the outer leaflet of the lipid-bilayers where GPI anchored proteins are also present through a covalently attached GPI structure in various cells.[67),68)] Recently a newly synthesized GPI-anchored protein was demonstrated to be localized in membrane subdomains enriched with both SM and glycosphingolipids.[69)] To address the possibility that GPI-anchored proteins interact with sphingolipids, we (K. Hanada, and his coworkers) constructed the transfectants expressing CD14, which is a GPI-anchored protein and well-known as the LPS receptor, from mutant SPB-1 and the parent CHO cells, and examined the effect of sphingolipid deficiency on CD14, especially in terms of its sensitivity to phosphatidylinositol-specific phospholipase C (PI-PLC). We found that CD14 expressed in mutant SPB-1 cells was hypersensitive to PI-PLC, compared with that in the parental CHO cells, when the cells were preincubated in a sphingolipid deficient medium at 39 °C. Furthermore, when the cells were exposed to fumonisin B1, an inhibitor of ceramide synthase, CD14 even in CHO cells became hypersensitive to PI-PLC. The PI-PLC hypersensitivity in the SPB-1 cells was almost completely suppressed when the cells were cultured in the presence of exogenous sphingosine or SM. Likewise, CD14 molecules in membranes prepared from the sphingolipid-deficient cells were more sensitive to PI-PLC than those from the parent CHO cells. These results indicated that deficiency in cellular SM caused the hypersensitivity of CD14 to PI-PLC, suggesting the specific interaction of not only CD14 molecule but also possibly all the other GPI-anchored proteins with SM within membranes in order to protect all the GPI-anchored proteins from the attack by PI-PLC.[66)]

5-2-2. Strong interactions among SM, cholesterol and GPI-anchored protein.[70)] In order to get further information on the specific interactions between SM and CD14 described above, we further constructed the transfectants expressing the different GPI-anchored protein, human placental alkaline phosphatase (PLAP) in mutant SPB-1 and also in CHO cells. By using these transfectants, we examined the effects of SM and/or cholesterol deprivation on the solubility of PLAP in Triton X-100, which we used as an internal indicator for the presence of such complexes tightly associated with SM, all the GPI-anchored proteins, and an additional factor, possibly cholesterol together within the membranes. The PLAP solubility in Triton X-100 from the membranes of the transfected CHO cells was only 10%, while that in Triton X-100 from the transfected mutant cell membranes deprived of both SM and cholesterol increased up to 50%. However this enhanced PLAP solubility from the transfected mutant cell membrane was again suppressed to the control level by metabolic complementation with exogenous sphingosine and cholesterol. The SM and cholesterol contents of the isolated membranes were changed independently, eliminating the possibility that SM deprivation induce a reduction in cellular cholesterol and enhanced PLAP solubility, and vice versa. We also know that no structural change occurred in PLAP molecule during SM and/or cholesterol deprivations; actually all the PLAP had the GPI-anchor moiety and there was no difference in the apparent molecular weight of the protein in the supernatant and the precipitated fractions of the detergent-treated membranes. In addition, the formation of caveolin in the isolated membranes was not significantly affected by SM and/or cholesterol depletion. These results clearly indicated that both SM, and cholesterol were involved in the insolubility of GPI-anchored proteins, and suggested that these lipids play important roles in formation of Triton X-100-insoluble complexes. Namely, SM has the strong affinity to cholesterol to make microdomains within the plasma membrane, so-called "the raft structure", a tightly associated structure, where we found the GPI-anchored proteins are specifically located for their functionings.[70)]

5-3. Regulation in biosynthesis of sphingomyelin (SM). Mammalian cells are primarily surrounded by the plasma membrane and have the different kind of intracellular organelles not only with a little bit of different phospholipid compositions in their membranes, but also with the specified enzymes for biosyntheses of the corresponding phospholipids. As described in the PS-biosynthesis, during or after the biosyntheses of membrane phospholipids, the transferring and sorting of phospholipids from their synthesized sites to their appropriate destinations are the most important events for membrane biogenesis in mammalian cells.

During the biosyntheses of sphingolipids in mammalian cells, first of all, ceramide is newly produced in the ER and transferred to the trans-Golgi regions, where it is further converted to SM by phosphatidylcholine ceramide cholinephosphotransferase (SM synthase). However, the mechanism underlying the "transferring of ceramide" remains to be clarified.

After my retirement from NIH of Japan, K. Hanada (the responsible scientist of our sphingomyelin (SM) project), and his coworkers continued this project. Actually his works were based on his wonderful ideas to develop the project further which finally led to the new finding of the responsible protein (the ceramide trafficking protein, abbreviated as "CERT") which could transfer the newly synthesized ceramide from the ER to Golgi apparatus for the biosynthesis of SM. This is the first, and the most valuable finding on the biogenesis of mammalian cell membranes and also in cell biology, leading to the general understanding on the presence of the specific lipid-transfer protein contributing to trafficking some (phospho-)lipids between or among the intracellular organelles during the biosyntheses of their final lipid products.

His beautiful work on this project has already been written by himself as a review article in this journal,[71] Proc. Japan Acad., Ser. B **86**, 426–437 (2010), and so if the readers have the great interest in his further development, please refer to his review article written more precisely.

However, here I would like to briefly summarize the further striking progress for ceramide trafficking by K. Hanada and his coworkers.

i) First of all, he used the cytolytic toxin named as lysenin derived from the earthworm, which specifically binds to SM present in the outer leaflet of lipid bilayers in the plasma membranes and lyses the mammalian cells.[72] So if we could get the lysenin-resistant CHO cells, their SM contents are not or only slightly present in their plasma membranes. Actually from the lysenin-resistant CHO cells, he could easily get not only the mutant cells defective in serine-palmitoyl transferase (named as LY-B, the same type of mutant cells of our former SPB-1 cells), but also the new mutant cells with normal serine-palmitoyl transferase but defective in ceramide trafficking from the ER to Golgi apparatus (named as LY-A).[73] The LY-B cells were used for his further works on serine-palmitoyl transferase, the results of which were summarized in his review article.[74] The LY-A cells were used for further analyses of the ceramide trafficking.

ii) The second was to devise the new method for detection of ceramide trafficking from the RE to Golgi apparatus by using both the parent CHO cells and the mutant LY-A cells with the perforated plasma membranes. Under such conditions both the ER and Golgi apparatus are functionally intact within the cells but almost all the cytosolic soluble proteins and also the much small-moleculer compounds were leaked out from the cells, leading to the conclusion that the parental soluble protein is essential for the ceramide trafficking but the soluble proteins of LY-A cells is not.[75]

iii) However, after treating the LY-A cells with the perforated plasma membranes by brefeldin A, which has the ability to merge or fuse the ER with Golgi apparatus, he observed the conversion of ceramide to SM even in the mutant LY-A cells with the non-functional ceramide-trafficking protein, indicating under such a special experimental condition no ceramide-trafficking protein is essential.

iv) By using the perforated, semi-intact cells, he succeeded in reconstitution of the intracellular ceramide-trafficking system by adding both ATP and the parental cytosolic soluble protein; namely, the de novo synthesized ceramide in the ER was converted to SM in Golgi apparatus.[75]

v) By utilizing the special character of the SM-deficient mutant (LY-A cells) which is extremely sensitive to the cholesterol adsorbent, methyl beta cyclodextrin, he succeeded in isolation of the cDNA that rescues LY-A cells.

All these results described above finally led to the new finding of the specific protein for ceramide-trafficking (abbreviated as "CERT"), which is contributing to ceramide trafficking from the ER to Golgi apparatus during the biosynthesis of SM. "CERT" is a hydrophilic 68-KDa protein, and responsible for ceramide trafficking with the specific

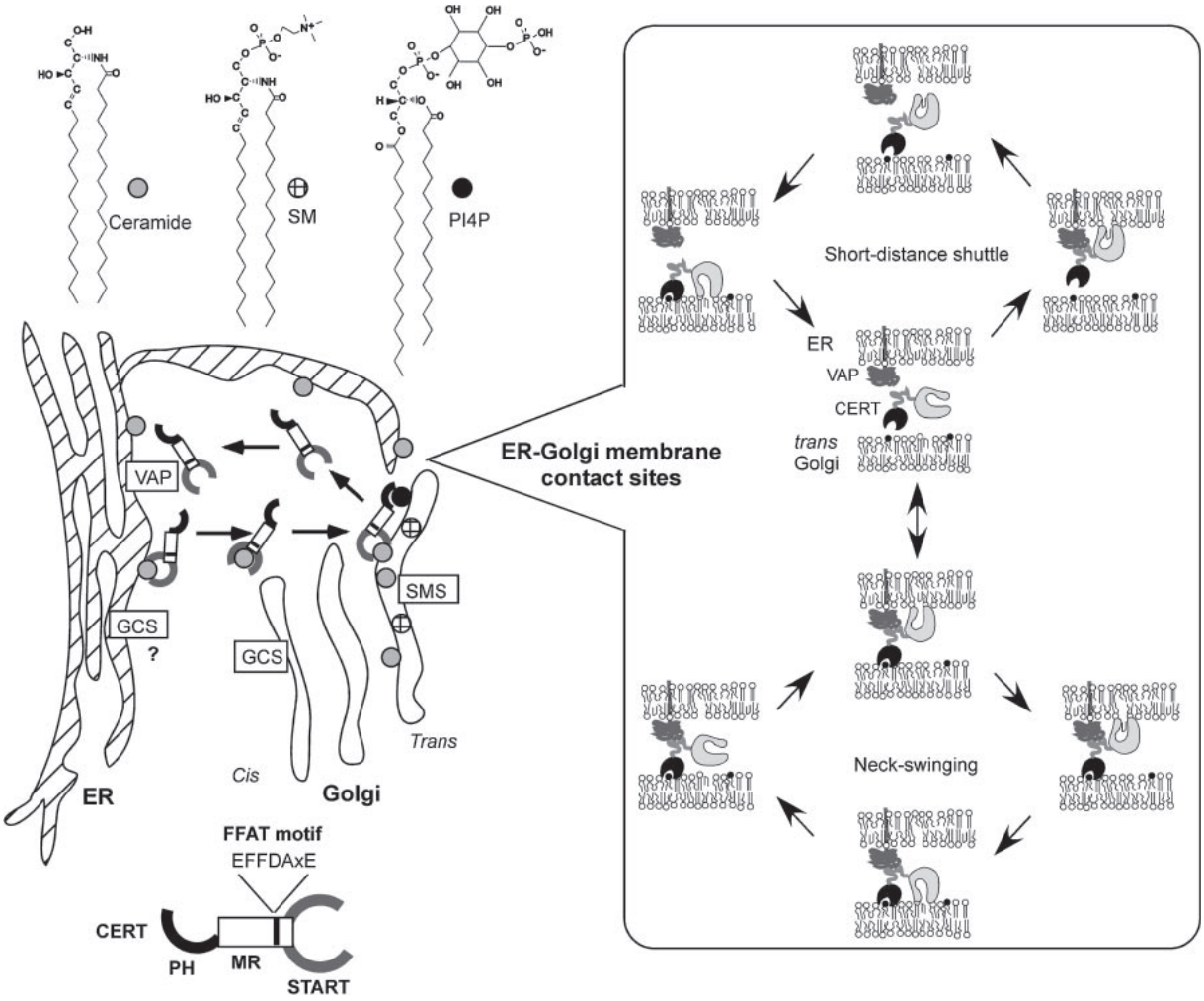

Fig. 10. A model of CERT-mediated trafficking of ceramide from the ER to the *trans* Golgi region.[71] SMS, SM synthase; GCS, GlcCer synthase. Inset, efficient trafficking of ceramide through short-distance shuttling by CERT or through the 'neck-swinging' movement of the START domain might occur at the sites of contact between the ER and *trans* Golgi cisternae.[71]

functional domains within the molecule, for not only the trafficking of ceramide from the ER (by using its C-terminal motif, START domain of "CERT" for picking up ceramide in the ER) to the Golgi (by its N-terminal motif, PH domain of "CERT" for binding to phosphatidylinositol 4-phosphate (PI-4P) located in Golgi apparatus), but also for the recycling of "CERT" from the Golgi to the ER[71),75)] (by FFAT motif in the middle region of "CERT" for binding to VAP in the ER).

For the readers convenience, finally I would like to cite "his proposed model for functions of CERT" shown as Fig. 10 in this review.[71)]

His work is at present still expanding not only as the basic research but also as exploitation for the medical application.

Perspectives

Our technology used for isolation of mammalian cell mutants are very useful for understanding not only the regulations of phospholipid biosynthesis but also the biological roles of membrane phospholipids. Thus the target cells could be further expanded to plant and vegetable cells too.

I hope these various variant cells could contribute to the promotion not only for the basic sciences including other area of cell biology but also for the medical applications, and finally for health and welfare of the human beings.

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Finally as a Director, I have to thank to all the members of my laboratory including the above described scientists and others for their faithful contribution not only to the above described science but also to the obligatory service headed by Miss S. Hara for the National Certificate Examination of the Biologics including antibiotics from the standpoint of chemist prior to their marketing, not only to Japan but also to all over the world.

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Profile

Yuzuru Akamatsu was born in Zushi-shi, Kanagawa Prefecture in 1933. He is a graduate of the University of Tokyo (B.S., Pharm. Sci., 1958, Master Degree, in Chemistry, 1960). Then he moved to NIH of Japan, where he got the special training for handling *Mycobacterium tuberculosis*. One year after, he got back to Prof. S. Nojima's Laboratory, supervised by Prof. D. Mizuno, Dept. Pharm. Sci., the Univ. of Tokyo as a Faculty-Assistant, where he met his life-work project on "membrane phospholipids" in bacterial cells and started his research career at the very early stage of their Biochemistry and Cell Biology. After he got his Ph. D. degree from the Univ. of Tokyo (1965), he spent the University of Chicago, as a research associate of the University and also as an US-Japan Fulbright Exchange Scholar, supervised by Prof. John H. Law, where he made the fruitful, scientific achievements on the fatty acyl moieties of phospholipids in *Mycobacteria*.

After getting back to Dr. Nojima's laboratory, NIH of Japan as a Chief of Biochemistry Section (1970), he expanded his scientific field in combination of biochemistry with genetics. During his stay in the late Prof. George J. Schroepfer Jr.'s laboratory (Rice University in Houston as a Visiting Professor), he was promoted to Director of Depart. of Chem., NIH of Japan (NIID of Japan).

As a Director (1976), he changed the research target of his phospholipid project to mammalian cells. It was the very important moment of his research career, because, apart from the classical culturing technology of "so-called the tissue culture era", his group devised the up-to-the-second technology for isolation of the mutant cells from the mammalian cultured cells. By using this technology, his group successfully isolated the various mammalian cell mutants defective in phospholipid biosyntheses, which led to the new finding in membrane biology, a part of which is summarized in this review. These findings gave him the chances to present his work at the international conferences including the Gordon Research Conferences as an invited speaker. In addition, his group also isolated the lipopolysaccharide (LPS)-resistant mutant, with defective LPS binding, of the cultured macrophage-like cells, which led not only to the new finding of the LPS receptor protein on the macrophages, but also to the development of the immuno-potentiator. After retirement from NIID of Japan (1994), he learned how to develop the antibiotics; in Meiji Seika Kaisya, Ltd., and also in the Microbial Chemistry Research Foundation, Tokyo.

He is now the Honorary Member of NIID of Japan (since 1994), also the Honorary Members of the American Society of Biochemistry and Molecular Biology, U.S.A. (since 1995), and of the Japanese Conference on the Biochemistry of Lipids (since 2000). He is a member of the Board of Trustee of the Japanese Biochemical Society.

He was laurelled with the Academic Prize with Gold Medal from the Pharmaceutical Society of Japan (1993). He was further awarded with the Order of the Sacred Treasure, Gold Rays with Rosette from Japan (2004).