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JB Reflections and Perspectives

Shigeru Tsuiki: a pioneer in the research fields of complex carbohydrates and protein phosphatases

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Dr Tsuiki made three major contributions during his illustrious career as a biochemist. First, he developed the procedure for mucin isolation from bovine submaxillary glands. His work became the basis for mucin biochemistry. Second, he identified four distinct molecular species of mammalian sialidase. Subsequent studies based on his work led to the discovery that sialidase plays a unique role as an intracellular signalling factor involved in the regulation of a variety of cellular functions. Finally, he established the molecular basis for the diversity of mammalian protein phosphatases through protein purification and molecular cloning. His work prompted the functional studies of protein phosphatases.

Keywords: cancer research/complex carbohydrate/protein phosphatase/sialidase.

Abbreviations: CMP-NeuAc, CMP-*N*-acetylneuraminic acid; DSP, dual specificity protein phosphatase; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; GFAT, glutamine:fructose-6 phosphate amidotransferase; GP, glycogen phosphorylase; GS, glycogen synthase; IL, interleukin; JNK, c-Jun N-terminal kinase; MKP, mitogen-activated protein kinase phosphatase; NF-κB, nuclear transcription factor-κB; NIPP, nuclear inhibitor of PP1; PP, protein phosphatase; PTP, protein tyrosine phosphatase; SAPK, stress-activated protein kinase; SH, src homology; TAK, transforming growth factor-activated kinase; TGF, transforming growth factor; UDP-GlcNAc, UDP-*N*-acetylglucosamine; VAP, vesicle-associated membrane protein-associated protein.

Dr Shigeru Tsuiki graduated from Tohoku University Medical School (Japan) in 1949 (Fig. 1). In 1951, he began his career as a biochemist as a graduate student in the laboratory of Dr Hajime Masamune, in the Department of Biochemistry at Tohoku University Graduate School of Medicine. After obtaining his PhD degree in 1956, he was appointed to assistant professor and then associate professor in Dr Masamune's lab. He moved to the University of Alabama Birmingham Medical School (USA) as a visiting scientist in the laboratory of Dr Ward Pigman. In Dr Pigman's lab he made a very important contribution to the research field of mucin; he established a procedure for the isolation of mucin from bovine submaxillary glands. Three years later, in 1963, he returned to Tohoku University and was promoted to Professor of Biochemistry in the Institute for Tuberculosis, Leprosy and Cancer. Throughout his 27 year career as a professor, he made pioneering works in the research fields of biochemistry, complex carbohydrates and protein phosphatases. After retirement from Tohoku University, Dr Tsuiki became a professor in Tohoku Pharmaceutical University in 1990, where he continued to contribute to the education of young students and scientists. The authors of this article joined Dr Tsuiki's lab at Tohoku University as graduate students and later worked as research staffs.

Studies of Complex Carbohydrates

Development of an ideal method for mucin purification from bovine submaxillary glands

Dr Tsuiki began his research career studying carbohydrate chemistry of *N*-glycoside mutarotation (*1*) as a graduate student in Dr Masamune's laboratory. Upon completion of his PhD degree, he joined the laboratory of one of the leading American carbohydrate chemists, Dr Pigman. Under his guidance Dr Tsuiki made an outstanding contribution to glycoconjugate chemistry by developing a convenient method for the preparation of bovine submaxillary mucin in a relatively undegraded and homogenous state using cetyltrimethylammonium bromide (*2*, *3*). This innovative method greatly contributed to the elucidation and characterization of peptides and saccharide structures of mucin. The best mucin obtained consisted of about 35% peptide units and galactosamine and sialic acid units in equal molar ratios. In those days, his report was referred to as 'the Bible for submaxillary mucin' among researchers in Dr Pigman's laboratory. Dr Tsuiki's interest in sialic acid components of mucin saccharides continued and led to his subsequent studies of sialic acid-relating enzymes.

Characterization of the enzymes involved in sialic acid biosynthesis

Sialic acids, which occur primarily in terminal positions of carbohydrate portions of glycoproteins and glycolipids, are considered to play important roles in various biological processes largely in two ways; one related to their hydrophilic and acidic properties exerting physiochemical effects on the glycoconjugates, and the other as recognition sites, or in an opposing fashion, as masking sites. In the 1960s and 1970s, the subject of cell surface sialic acids appearing in cancer cells received a great deal of attention: a large number of studies suggested that the associated increase in negative surface charge and change in electrophoretic mobility was correlated with malignant properties of cancer cells. In the same era, isoenzyme studies were concentrated on elucidation of aberrant gene expression in carcinogenesis, with particular concern on enzymes of the glycolytic pathway. One such study presented evidence that in rat liver, the liver type isozymes of glycolytic pathway were replaced by other forms in hepatomas. With this background, after becoming a professor at Tohoku University, Dr Tsuiki speculated that such neoplastic alteration could also include sialic acid biosynthesis, and began to investigate this possibility as one of his cancer research projects. He focused on two key enzymes involved in sugar nucleotide biosynthesis (*4*): glutamine: fructose-6 phosphate amidotransferase (GFAT); and UDP-*N*-acetylglucosamine (UDP-GlcNAc) 2-epimerase. UDP-GlcNAc and CMP-*N*-acetylneuraminic acid (CMP-NeuAc) are considered important end products and substrates for glycosyltransferase transfer of GlcNAc and NeuAc (sialic acid), respectively, to glycoproteins and glycolipids. These enzymes demonstrated distinct behaviours during rat liver development (*5*) and in hepatomas with kinetics and chromatographic behaviour. UDP-GlcNAc 2-epimerase could be partially purified from normal rat liver and characterized (*6*), along with GFAT, for comparison with hepatoma (*7*) and regenerating liver. These works have often been quoted by subsequent researchers. Recently, important roles of the two enzymes in diseases have been described: GFAT overexpression is linked to insulin resistance and Type 2 diabetes, and UDP-GlcNAc 2-epimerase mutation can result in hereditary inclusion body myopathy or sialuria.

Neoplastic alteration of sialyltransferase

Altered glycosylation has been reported in cell surface glycoproteins of malignant cells (*8*, *9*), and an increase in sialylation often occurs at the termini of their sugar chains (*8*). Such alteration of glycolipids is also observed as a ubiquitous phenotype, associated with the appearance of tumour-associated antigens, aberrant adhesion, and inhibition of transmembrane signalling (*10*). However, drawing definite conclusions regarding physiological links between sialic acid content and malignant properties proved difficult due to conflicting experimental results. To cast further light on the causes of such aberrant sialylation and the consequences, the studies of Dr Tsuiki were then extended to sialyltransferase, transfering sialic acids from CMP-NeuAc into glycoproteins. Sialyltransferases were purified to near homogeneity for the first time using affinity elution from rat liver and 3′-methyl-4-dimethylaminoazobenzene-induced primary hepatomas (*11*), and asialofetuin sialyltransferase from Triton X-100 extracts of rat liver was resolved by phosphocellulose chromatography into two fractions, designated transferases I and II in the order of elution. When previously treated with *Arthrobacter ureafaciens* sialidase, the transferase I eluted at relatively similar position as the transferase II while no alteration occurred in II. Primary rat hepatomas were found to contain only a single asialofetuin sialyltransferase, identical to transferase I in chromatographic behaviour. Transferase II, as well as sialidase-treated I, could be sialylated auto-catalytically. Both enzymes formed (α2-6) sialylgalactoside linkages with asialoorosomucoid and with lactose as well as with asialofetuin. While indistinguishable immunologically, the transferases exhibited distinct heat stability and kinetic properties. Transferase I showed a three times higher affinity than II for CMP-NeuAc and for desialylated plasma membranes. These results suggested that the predominance of transferase I might be responsible for the increased sialylation of membrane glycoproteins previously reported for experimental hepatomas.

Two months later in the same year, Dr Paulson's group also reported purification and characterization of the β-galactoside (α2-6) sialyltransferase from rat liver in combination with the results on β-galactoside (α2-3) sialyltransferase (*12*). This group of long experience in the research field utilized sophisticated affinity chromatography on CDP-hexanolamine-agarose for purification of the enzymes, and Dr Tsuiki's group employed an easy CM-sepharose column with a special cytidine 5′-triphosphate affinity elution. These were the first reports for sialyltransferases highly purified from mammalian tissues.

Identification of multiple forms of mammalian sialidase

To further elucidate the significance and molecular mechanisms underlying altered sialylation, Dr Tsuiki decided to study mammalian sialidases, which regulate cellular sialic acid contents and function of glycoconjugates by desialylation. It is well known that the removal of sialic acid residues from glycoproteins and glycolipids, catalysed by sialidase, is the initial step in the catabolism of these glycoconjugates, with great influence on their biological functions. In the early 1980s to 1990s, based on biochemical approaches, Dr Tsuiki's group discovered evidence for the existence of four types of sialidase differing in their subcellular localization and enzymatic properties, including their substrate specificity (*13*–*15*). They were classified according to their major intracellular localization as intralysosomal, cytosolic and membrane-associated sialidases I and II. Several rat tissues, including the liver and brain, and even isolated rat hepatocytes were found to contain all the four types. Intralysosomal sialidase possesses narrow substrate specificity, such that only oligosaccharides and glycopeptides served as its substrates (*13*). Sialidase found in the cytosol, in

contrast, is able to hydrolyse glycoproteins and gangliosides at near neutral pH (*14*). Both sialidases are, however, distinct from the membrane-associated sialidases in that the latter required detergents for solubilization and preferentially hydrolyses gangliosides (*15*). Membrane sialidase I scarcely hydrolysed other substrates including oligosaccharides and glycoproteins, while membrane sialidase II acts on oligosaccharides, glycoproteins and even on the internal sialic acid residue of gangliosides GM2. Sialidase I proved to localize mainly in the plasma membranes and sialidase II predominantly in the mitochondrial–lysosomal membrane fractions. Biochemical characterization of the four forms of sialidase suggested that each might play a unique role depending on its particular subcellular localization and catalytic properties. It is important to note that recent progress in molecular characterization of sialidases has validated this hypothesis, with confirmation of enzymatic properties and subcellular localization (*16*).

To further elucidate their functional roles and expression mechanisms, Dr Tsuiki's group focused on isolating a sialidase gene. Most attempts to extensively purify mammalian sialidase had so far been unsuccessful, primarily due to the extreme instability and low activity of the enzymes. Of the four types of sialidases, Dr Tsuiki's group was able to purify the cytosolic sialidase to apparent homogeneity from rat liver cytosol, the first example for highly purified mammalian sialidase (*14*). Since a tissue distribution study showed activity to be about 16-fold greater in skeletal muscle than in liver, skeletal muscle sialidase was then purified for analysis of partial amino acid sequence. Although a number of sialidase genes from microorganisms were isolated, no cDNA encoding mammalian sialidase had been identified. After struggling for 7 years, Dr Tsuiki's group finally succeeded in cloning a cDNA-encoding cytosolic sialidase of rat skeletal muscle by applying the PCR, based on amino acid sequence data for the purified enzyme (*17*). The sequence contained two typical Asp blocks (-Ser-X-Asp-X-Gly-X-Thr-) and the Arg-Ileu-Pro sequence, which had been found in sialidase genes from some microorganisms, although the primary structures were not particularly similar overall. This was the first discovery regarding mammalian sialidase gene structure.

Present state and perspectives

Sialidase research has been continued in Taeko Miyagi's laboratory (Miyagi Cancer Centre Research Institute and then Tohoku Pharmaceutical University) from the particular viewpoint of importance to cancer. To determine potential causes and consequences of aberrant sialylation in cancer, structural and functional studies of mammalian sialidase were conducted. Since earlier works on rat liver sialidase directed by Dr Tsuiki suggested a link between membrane sialidase I and carcinogenesis, efforts were made to clone the sialidase gene. After failure in many trials, the sialidase was finally isolated from bovine brain in a highly purified form, and the gene was cloned based on the peptide sequence information (*18*). Catalysis by the recombinant sialidase was essentially specific for gangliosides, and its major subcellular localization proved to be the plasma membranes. This sialidase plays a role in neuronal function and is located in caveolae, closely associated with caveolin-1. Very recent studies have provided evidence that the sialidase plays an important role in cellular signalling through modulation of gangliosides and also probably through interaction with signalling molecules including rac-1, Grb-2 and epidermal growth factor receptor (EGFR) (*19*). As expected, the sialidase is markedly up-regulated in various human malignancies including colon (*20*), renal (*21*), ovarian and prostate cancers, where it may cause accelerated progression to malignant phenotypes by activating signalling pathways including EGFR, focal adhesion kinase, integrin-linked kinase, Shc and integrin β4, often found up-regulated in carcinogenesis.

The sialidases, localized predominantly in the lysosomes, cytosol and plasma membranes, have now been designated as Neu1, Neu2 and Neu3, and the fourth Neu4 has been characterized to possess relatively broad substrate specificity and be localized in mitochondria and endoplasmic reticulum, which is essentially consistent with the earlier biochemical findings in Dr Tsuiki's laboratory. These four types of human sialidases identified to date behave in different manners during carcinogenesis (*22*). Among them, Neu1 shows down-regulation in cancers, this promoting anchorage-independent growth and contributing to metastatic ability (*23*), while Neu3 exhibits marked up-regulation, resulting in suppression of apoptosis. Furthermore, Neu3 silencing has been shown to cause apoptosis without specific stimuli in many cancer cell lines, but interestingly non-cancerous cells showed no significant changes (*24*). Investigation of mammalian sialidases has clarified some of the molecular bases of aberrant sialylation, and alteration in sialidase expression may be a defining factor for cancer progression.

Starting from the investigation of sialic acid-relating enzymes in rat liver and hepatomas in Dr Tsuiki's laboratory, research in this area has now extended to human cancer, in the hope of providing valuable new information for cancer diagnosis and therapy. In particular, down-regulation of Neu3 expression by treatment with the specific siRNA, antibody or inhibitor may lead to prevention of cancer progression. Taking advantage of its limited effects on normal cells, targeted Neu3 inhibition leading to apoptosis in cancer cells could be effective for cancer therapy.

Studies of Protein Phosphatases

Study of the individuality of ascites hepatoma cells: a clue for protein phosphatase research

Studies of protein phosphatases by Dr Tsuiki developed from the research project that compared the biochemical properties of various rat ascites hepatoma cells. Previous studies had shown that although most rat ascites hepatoma cell lines contain very small amounts of glycogen (<3 μmols of glucose equivalent/g of wet tissue), a few cell lines contained about 20 times larger amounts of glycogen. Dr Tsuiki was interested in investigating the biochemical mechanism

regulating glycogen content in the ascites hepatoma cell lines, AH-66F with a high glycogen content (59 μmols of glucose equivalent/g of wet tissue) and AH-130 with low glycogen content (1.8 μmols of glucose equivalent/g of wet tissue). His studies suggested that cellular glycogen content was controlled in part by glycogen itself, in a negative feedback manner, such that synthesized glycogen inhibited glycogen synthesis; this feedback mechanism was much less effective in AH-66 F than in AH-130 (*25*). This work motivated Dr Tsuiki to investigate the regulation of glycogen synthesis by protein phosphatases.

Discovery of multiple protein phosphatases with distinct substrate specificity in rat liver

It had already been established that the enzymes responsible for glycogen synthesis and glycogen degradation were glycogen synthase (GS) and glycogen phosphorylase (GP), respectively. GS catalyses the transfer of glucose from UDP-glucose to primer glycogen, while GP catalyses the phosphorolysis of glycogen, generating glucose-1-phosphate from glycogen. Both GS and GP were shown to be regulated by phosphorylation and dephosphorylation. Thus, dephospho-GS (GSI) is the active form which is inactivated by phosphorylation on Ser/Thr residues catalysed by protein kinases, whereas phospho-GS (GSD) is activated by dephosphorylation catalysed by protein phosphatases. In contrast, dephospho-GP (GPb) is the inactive form which is phosphorylated and activated by phosphorylase kinase, while phospho-GP (GPa) is dephosphorylated and inactivated by protein phosphatase.

In relation to the protein phosphatase(s) responsible for dephosphorylation of GPa, Brandt *et al*. (*26*) reported that a 34 kDa protein purified by 80% ethanol treatment of rabbit muscle extracts at room temperature readily dephosphorylayed GPa *in vitro*, and proposed that this protein phosphatase was a general protein phosphatase responsible for the dephosphorylation of a variety of phosphoproteins. However, no information was obtained regarding the ability of the 34 kDa phosphatase to dephosphorylate and activate GS. Therefore, Dr Tsuiki decided to study the enzymatic properties of rat liver GS phosphatase, which would be helpful for understanding the mechanism of feedback regulation of glycogen synthesis by glycogen.

The major difficulty of studying the protein phosphatase responsible for activation of GS was that GS contained multiple sites of phosphorylation, and the exact phosphorylation site(s) required for inactivation of GS had not yet been determined. In addition, GS was tightly associated with glycogen particles which inhibited the accurate assessment of GS phosphatase activity. Dr Tsuiki developed a method to solubilize rat liver GS from glycogen particle using DE52 column chromatography (*27*). He used solubilized GS as the substrate to determine the rat liver GS phosphatase activity. The activity of GS phosphatase was determined by the ability of phosphatase to increase GS activity in the absence of glucose-6-phosphate. Success in setting up of GS phosphatase assay system enabled Dr Tsuiki to start the GS phosphatase project. He also used GPa and ^{32}P-histone as the substrates of protein phosphatase to determine the substrate specificity of the phosphatase molecule(s).

According to Dr Tsuiki's strategy to purify protein phosphatases in their native forms, he avoided drastic treatment of the rat liver extracts as much as possible and utilized several successive column chromatographies. The chromatography on DE52 column revealed that elution profile of the major GS phosphatase activity was different from that of GP phosphatase activity. The GS phosphatase and the GP phosphatase activities eluted at 0.26 M NaCl (peak I) and 0.39 M NaCl (peak II), respectively (*27*). Both peaks I and II had histone phosphatase activities. Peak I was subsequently separated into two peaks (IA and IB) using AH-Sepharose column chromatography (*28*). Peaks IA and IB showed relatively high specificity for GS and histones, respectively, while peak II showed relatively high specificity for GPa. Estimation of the molecular weights of the three protein phosphatases by Sephadex G-200 column chromatography indicated that sizes of peaks IA, IB and II were 69, 300 and 160 kDa, respectively (*28*). This work was the first demonstration of the existence of multiple protein phosphatase molecules with differing molecular weights and substrate specificities in mammalian cells.

Purification and molecular properties of three different protein phosphatases from rat liver

Following the discovery of three distinct protein phosphatases separated by DE52 and AH-Sepharose column chromatographies, Dr Tsuiki spent a few years to purify each of these three protein phosphatases to near homogeneity without disrupting their molecular weights and substrate specificities. Starting from about 300 g of rat livers, he finally succeeded in the purification of several micrograms of phosphatases IA, IB and II using combinations of DE-52, AH-Sepharose, Protamine-Sepharose, Histone-Sepharose and Sephadex G-200 column chromatographies. The molecular sizes of the purified phosphatases IA, IB and II were 45, 260 and 160 kDa, respectively (*29–31*). Analyses of subunit composition of these three phosphatases revealed that phosphatase IA was a monomeric protein, while phosphatases IB and II were oligomeric proteins composed of three (α, β and γ) and two (α and β) subunits, respectively (*29–31*). Molecular sizes of α, β and γ subunits were 35, 69 and 58 kDa, respectively. Studies of metal ion requirement of the purified phosphatases revealed that phosphatase IA required Mg^{2+} or Mn^{2+} for its activity, whereas no metal ion was required for phosphatases IB and II activities. These works were the first purification and subunit identification of different molecular forms of mammalian protein phosphatases. Protein phosphatase IA was subsequently classified as protein phosphatase (PP)2C, while protein phosphatases IB and II were designated two different holoenzymes of PP2A by Philip Cohen (University of Dundee, UK) (*32*).

Purification of a protein tyrosine phosphatase with src homology region 2

Since the identification of src as a protein tyrosine kinase, the identification of molecular features of

putative protein tyrosine phosphatases (PTPases) has attracted the interest of protein phosphatase researchers. Dr Tsuiki decided to expand his research scope and made efforts to purify and determine the biochemical properties of PTPases. Utilizing three proteins plus tyrosine-glutamate copolymer as substrates, he partially purified four different PTPases from rat liver cytosol. Of the four PTPases, tentatively termed L1, L2, L3 and L4, PTPase L1 was purified to apparent homogeneity by a procedure involving several different chromatographies and it showed a major protein band of 67 kDa by SDS/PAGE (*33*). Amino acid sequence analysis of the purified PTPase L1 revealed that this protein had 55 and 68% amino acid sequence homology with the catalytic domain and the two adjacent copies of src homology 2 (SH-2) domain of PTP1C (*34*), respectively. Based on these results Dr Tsuiki concluded that PTPase L1 was a novel PTPase with two SH-2 domains in the molecule, which was also termed SH-PTP2 (*35*).

Molecular cloning of protein phosphatase 2C (IA)

In early 1980, a new wave of basic biological science, molecular biology, began to overwhelm the research field of biology. This prompted Dr Tsuiki to take a molecular biological approach to his research project. He purified PP2C (50 μg) from rat livers using conventional purification procedures and analysed the amino acid sequences of the peptides obtained by tryptic digestion of the purified protein in collaboration with Dr Joseph Larner of University of Virginia (USA). Based on the sequence of two oligopeptides, they synthesized mixed oligonucleotide probes and screened a cDNA library in collaboration with Dr Kevin R. Rynch of University of Virginia and succeeded in isolation of a cDNA clone encoded PP2C, which they used to successfully express the functional recombinant PP2C protein in *Escherichia coli* (*E. coli*) (*36*, *37*). This was the first molecular cloning and expression in *E. coli* of a PP2C family member. The PP2C molecule encoded by the cDNA isolated by Dr Tsuiki and colleagues was later designated PP2Cα (*38*).

Present state and perspectives

After the retirement of Dr Tsuiki from Tohoku University, the protein phosophatase research he initiated has been continued in Kunimi Kikuchi's lab (Hokkaido University, Japan) and Shinri Tamura's lab (Tohoku University) as described below.

Studies by Kunimi Kikuchi's group. Kikuchi's group have investigated the structure, function and regulation of three protein phosphatase families, protein serine/threonine phosphatase (PP), protein tyrosine phosphatases (PTPases) and dual-specificity protein phosphatases (DSPs), to elucidate the roles of protein phosphatases in neoplasmic alterations and in the immune system.

The full coding sequence of a Type 1 protein phosphatase catalytic subunit α (PP1α) was isolated (*39*), and an 8 kb genomic fragment of the 5′-flanking region of the gene encoding PP1α was cloned (*40*). Transcription of PP1α was shown to be controlled at the high GC region and activated in ascites hepatomas. Additionally, increased expression of nuclear inhibitor of PP1 (NIPP-1) mRNA was positively correlated with a malignant phenotype in rat hepatomas (*41*). Recent studies revealed that NIPP-1 functioned as a molecular sensor for PP1 to recognize phosphorylated spliceosome-associated protein 155 (*42*). Studies were also conducted to assess the structure–activity relationship on new phosphatase inhibitors containing thyrsiferyl 23-acetate, tautomycin and related compounds (*43*).

Expression patterns of 10 PTPase genes were investigated in regenerating livers and ascites hepatomas. From these studies, a clone of a novel cytoplasmic protein tyrosine phosphatase PTPε (PTPεC) was isolated, in addition to a transmembrane isoform (PTPεM) (*44*). It was suggested that different promoters control the expression of PTPεM and PTPεC during macrophage differentiation (*45*). PTPεC inhibits Jak-STAT signalling and differentiation induced by interleukin-6 and leukaemia inhibitory factor in M1 leukaemia cells (*46*).

Novel low-molecular-mass DSPs containing TMDP, LDP-2, LDP-3 and LDP-4 were cloned and characterized. Mitogen-activated protein kinase phosphatese-7 (MKP-7), a novel phosphatase of DSPs, was found to function as a shuttle protein (*47*). Activation of extracellular signal-regulated kinase (ERK) induces phosphorylation of MKP-7, a c-Jun N-terminal kinase (JNK)-specific phosphatase, at Ser-446 (*48*). Phosphorylation of Ser-446 determines stability of MKP-7 (*49*).

Studies by Shinri Tamura's group. Multiple cDNAs encoding proteins with high similarity of amino acid sequences with PP2Cα have been isolated following the molecular cloning of PP2Cα by Dr Tsuiki. To date the PP2C gene family is known to be composed of 14 different genes. This is in contrast to the small number of genes encoding the catalytic subunits of other protein Ser/Thr phosphatase families, such as PP1, PP2A and PP2B (3, 2 and 2 genes, respectively). In addition to PP2Cα, 3 (PP2Cε, PP2Cζ, and PP2Cη) of the 14 family members were initially cloned by the research group of Tsuiki/Tamura (*36*, *50–53*). A homology search indicated that the PP2C family has a unique evolutionary origin, while the catalytic proteins of PP1, PP2A and PP2B originated from a common molecular ancestor. The unique molecular evolution of PP2C raises the possibility that the PP2C family plays a unique physiological role in the regulation of cellular functions.

Molecular genetic studies using budding yeast had previously shown that osmotic stress induces transient activation of the Hog 1 signalling pathway, which corresponds to the stress-activated protein kinase (SAPK) signalling pathway in mammalian cells. Activation of Hog 1 induces glycerol synthesis that is negatively regulated by Ptc1, an orthologue of mammalian PP2C (*54*). Tamura's group determined that 2Cα, 2Cβ or 2Cε ectopically expressed in mammalian cells selectively suppresses the stress-induced activation of p38 and JNK, but had no effect on the mitogen-induced ERK activation (*50*, *55*, *56*). Investigation of the

Fig. 1 Dr Shigeru Tsuiki.

mechanism of the 2Cβ- and 2Cε-mediated suppression of the SAPK pathway revealed that both 2Cβ and 2Cε directly inactivate transforming growth factor (TGF)-β activated kinase (TAK)1, a mitogen-activated protein kinase kinase kinase which lies upstream of p38 and JNK (*50*, *56*, *57*). In response to stress, TGF-β, or interleukin (IL)-1, TAK1 is phosphorylated at multiple sites, and at least four sites are involved in the regulation of the TAK1 activity. Subsequent studies of functional differences between 2Cε and 2Cβ suggested that these two phosphatases inactivate TAK1 by dephosphorylating two different sites of TAK1.

It has been well established that, in many cell types, not only SAPKs but also nuclear transcription factor-κB (NF-κB) lie downstream of TAK1. Studies of the possible functional role of 2Cη in the regulation of stress response of cells performed by Tamura's group revealed that ectopic expression of 2Cη inhibited the IL-1-induced activation of NF-κB (*53*). Further studies indicated that 2Cη inhibits the IL-1-NF-κB signalling pathway by selectively dephosphorylating IKKβ, a protein kinase lying between TAK1 and NF-κB (53). Involvement of 2Cα and Wip1, another PP2C family member, in the regulation of SAPK pathway has also been reported by Takekawa *et al.* (*58*, *59*). Moreover, the participation of a large family of plant PP2C in stress response has also been reported (*60*). Taken together, these studies suggest that PP2C as a family plays a unique role in crisis management of cells.

Subsequent studies by Tamura's group indicated that 2Cε is an endoplasmic reticulum (ER) transmembrane protein with a N-terminal transmembrane domain and a C-terminal catalytic domain facing the cytoplasm (*61*). Studies of the possible novel role of 2Cε indicated that 2Cε associates with vesicle-associated membrane protein-associated protein (VAP)-A on the ER (*61*). VAP-A is an ER resident integral membrane protein that is involved in the recruitment of lipid-binding proteins, such as the ceramide transport protein CERT, to the ER membrane. It was demonstrated that the 2Cε associates with VAP-A, then dephosphorylates and activates CERT. Activated CERT then transports ceramide to the golgi apparatus where it promotes sphingomyelin synthesis (*61*).

Previous studies of ER functions have revealed that the ER is a multifunctional organelle which coordinates protein folding, lipid biosynthesis and calcium storage and release. Preliminary studies by Tamura's group have suggested that 2Cε participates not only in regulation of lipid metabolism but also in regulation of other ER functions. Currently, their major focus is the clarification of the roles of 2Cε as a multifunctional regulator of ER functions. Over time, these studies will potentially contribute to the understanding of the molecular basis of the pathology of the ER malfunction-related diseases.

Conflict of interest
None declared.

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