

Medical Proteomics Laboratory

疾患プロテオミクスラボラトリー

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The mission of our laboratory is to develop advanced technologies for integrative proteomic analyses from a physicochemical, structural and systems biology point of view. Currently, we mainly focus on functional protein-protein interaction networks related to a variety of diseases including cancer and infection. We are also engaged in collaborative researches regarding mass spectrometry and electron microscopy, which have made a substantial contribution to many scientific achievements.

<Group I>

1. Global characterization of the proteome and phosphoproteome in human glioblastoma initiating cells by high-resolution mass spectrometry

Hiroko Kozuka-Hata, Tomoko Hiroki, Ryo Koyama-Nasu¹, Kouhei Tsumoto, Jun-ichiro Inoue, Tetsu Akiyama¹ and Masaaki Oyama: ¹Laboratory of Molecular and Genetic Information, Institute of Molecular and Cellular Biosciences, University of Tokyo

Glioblastoma is one of the most common and aggressive brain tumors with the median survival of twelve months after diagnosis. Despite extensive studies of this malignant tumor, the outcomes of the treatment have not significantly improved over the past decade. To elucidate the underlying mechanisms of its tumorigenicity, we performed parallel analyses of the comprehensive proteome and phosphoproteome in glioblastoma initiating cells that are widely recognized as key players in showing resistance to chemotherapy and radiation. Using

high-resolution nanoflow LC-MS/MS (LTQ Orbitrap Velos) in combination with GELFREE™ 8100 fractionation system, we identified a total of 8,856 proteins and 6,073 phosphopeptides, respectively. Global protein network analysis revealed that the molecules belonging to ribosome, spliceosome and proteasome machineries were highly enriched at the proteome level. Our in-depth phosphoproteome analysis based on two fragmentation methodologies of CID and HCD detected various phosphorylation sites on neural stem cell markers such as nestin and vimentin, leading to identification of thirty-six phosphorylation sites including eleven novel sites of nestin protein. The SILAC-based quantitative analysis showed that 516 up-regulated and 275 down-regulated phosphorylation sites upon epidermal growth factor stimulation. Interestingly, the phosphorylation status of the molecules related to mTOR signaling pathway was dynamically changed upon EGF stimulation. More intriguingly, we also identified some novel phosphopeptides encoded by the undefined sequence regions of the human transcripts, which could be regulated upon external stimulation in glioblastoma initiating cells. Our result unveils an expanded diversity of the

regulatory phosphoproteome defined by the human transcriptome.

2. System-wide perturbation of the proteome and phosphoproteome dynamics in glioblastoma stem cells through mTOR signaling inhibition

Hiroko Kozuka-Hata, Tomoko Hiroki, Ryo Koyama-Nasu¹, Kouhei Tsumoto, Jun-ichiro Inoue, Tetsu Akiyama¹ and Masaaki Oyama.

As glioblastoma is the most common and aggressive brain tumor with poor prognosis, systematic elucidation of signaling networks causally linked to the tumorigenesis is very crucial for developing more effective treatments for this intractable cancer. In our previous study, we applied a high-resolution mass spectrometry-based proteomics technology in combination with SILAC quantitative methods to understand EGF-dependent phosphoproteome dynamics in patient-derived glioblastoma stem cells. We demonstrated that the phosphorylation levels of the representative mTOR signaling molecules such as RPS6 and PRAS40 were dramatically up-regulated upon EGF stimulation. As EGFR signaling has been reported to play a pivotal role in regulating the maintenance of cancer stem cells, we next carried out mTOR inhibitor-dependent signaling perturbations to unravel stemness-related pathways at the network level.

In the present study, we identified a total of 3,726 proteins including 49 up-regulated and 436 down-regulated factors by Torin 1 treatment. Interestingly, we found that one of the well-known cancer stem cell markers was significantly down-regulated through mTOR signaling inhibition. Our in-depth phosphoproteome analysis also led to identification of 6,250 unique phosphopeptides derived from 2,221 proteins and unveiled a variety of dynamic changes regarding phosphorylation levels of cancer and neural stem cell markers in a comprehensive manner. The integrative view of the mTOR inhibitor-dependent proteome and phosphoproteome dynamics in glioblastoma stem cells presents us with further prospects towards understanding previously unrecognized regulations at the system level.

3. Integrative Network Analysis Combined with Quantitative Phosphoproteomics Reveals Transforming Growth Factor-beta Receptor type-2 (TGFB2) as a Novel Regulator of Glioblastoma Stem Cell Properties

Yuta Narushima, Hiroko Kozuka-Hata, Ryo Koyama-Nasu¹, Kouhei Tsumoto, Jun-ichiro Inoue, Tetsu Akiyama¹ and Masaaki Oyama.

Glioblastoma is one of the most malignant brain tumors with poor prognosis and their development and progression are known to be driven by glioblastoma stem cells. Although glioblastoma stem cells lose their cancer stem cell properties during cultivation in serum-containing medium, little is known about the molecular mechanisms regulating signaling alteration in relation to reduction of stem cell-like characteristics. To elucidate the global phosphorylation-related signaling events, we performed a SILAC-based quantitative phosphoproteome analysis of serum-induced dynamics in glioblastoma stem cells established from the tumor tissues of the patient. Among a total of 2876 phosphorylation sites on 1584 proteins identified in our analysis, 732 phosphorylation sites on 419 proteins were regulated through the alteration of stem cell-like characteristics. The integrative computational analyses based on the quantified phosphoproteome data revealed the relevant changes of phosphorylation levels regarding the proteins associated with cytoskeleton reorganization such as Rho family GTPase and Intermediate filament signaling, in addition to transforming growth factor- β receptor type-2 (TGFB2) as a prominent upstream regulator involved in the serum-induced phosphoproteome regulation. The functional association of transforming growth factor- β receptor type-2 with stem cell-like properties was experimentally validated through signaling perturbation using the corresponding inhibitors, which indicated that transforming growth factor- β receptor type-2 could play an important role as a novel cell fate determinant in glioblastoma stem cell regulation.

4. Quantitative phosphoproteomics-based molecular network description for high-resolution kinase-substrate interactome analysis

Yuta Narushima, Hiroko Kozuka-Hata, Kouhei Tsumoto, Jun-ichiro Inoue and Masaaki Oyama.

Phosphorylation-dependent cellular signaling is known to play a diverse role in regulating multiple cellular processes such as proliferation, differentiation and apoptosis. Recent technological advances in mass spectrometry-based phosphoproteomics have enabled us to measure network-wide signaling dynamics in a comprehensive and quantitative manner. As conventional protein-protein interaction (PPI) information-based network analysis is insufficient to systematically analyze phosphorylation site-dependent complex interaction dynamics, here we develop and evaluate a platform to provide a high-resolution molecular network description for kinase-substrate interactome analysis. In this study, we developed a Cytoscape-based bioinformatical platform named "Post Translational Modification mapper (PTMapper)" to integrate PPI data with

publicly available kinase-substrate relations at the resolution of phosphorylated amino acid residues. The previous phosphoproteome data on EGF-induced cellular signaling in glioblastoma stem cells was applied to evaluate our platform, leading to discovery of phosphorylation-dependent crucial signaling modulation in the p70S6K1-related pathway. Our study revealed that high-resolution cellular network description of phosphorylation-site dependent kinase-substrate signaling regulation should accelerate phosphoproteomics-based exploration of novel drug targets in the context of each disease-related signaling.

5. System-level analysis of CagA-dependent signaling network dynamics by *Helicobacter pylori* infection

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The signal transduction system within a cell regulates complex biological events in response to bacterial infection. The previous analyses of cell signaling in *Helicobacter pylori*-infected gastric epithelial cells have revealed that CagA, a major virulence factor of *Helicobacter pylori*, is delivered into cells via the type IV secretion system and perturbs signaling networks through the interaction with the key signaling molecules such as SHP-2, Grb2, Crk/Crk-L, Csk, Met, and ZO-1. Although the biological activity of tyrosine-phosphorylated CagA has intensively been studied, system-wide effects of the virulence factor on cellular signaling have yet to be analyzed. Here we performed time-resolved analyses of phosphoproteome and CagA-interactome in human gastric AGS cells by CagA-positive/negative *Helicobacter pylori* infection. Our highly sensitive nanoLC-MS/MS analyses in combination with the Stable Isotope Labeling by Amino acids in Cell culture (SILAC) technology defined CagA-dependent perturbation of signaling dynamics along with a subset of CagA-associated possible modulators on a network-wide scale. Our result indicated that the activation level of the phosphotyrosine-related signaling molecules in AGS cells was suppressed overall in the presence of CagA during *Helicobacter pylori* infection. As *Helicobacter pylori* infection plays pivotal roles in the progression of gastric diseases including carcinogenesis, a comprehensive and fine description of the signaling dynamics would serve as a fundamental platform to theoretically explore for the potential drug targets through analyzing the regulatory mechanisms at the system-level.

6. Mass spectrometry-based annotation of the human short ORFeome

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In parallel with the human genome projects, human full-length cDNA data has also been intensively accumulated. Large-scale analysis of their 5'-UTRs revealed that about half of these had a short ORF upstream of the coding region. Experimental verification as to whether such upstream ORFs are translated is essential to reconsider the generality of the classical scanning mechanism for initiation of translation and define the real outline of the human proteome. Our previous proteomics analysis of small proteins expressed in human K562 cells provided the first direct evidence of translation of upstream ORFs in human full-length cDNAs (Oyama et al., *Genome Res*, 14: 2048-2052, 2004). In order to grasp an expanded landscape of the human short ORFeome, we have performed an in-depth proteomics analysis of human K562 and HEK293 cells using a two-dimensional nanoLC-MS/MS system. The results led to the identification of eight protein-coding regions besides 197 small proteins with a theoretical mass less than 20 kDa that were already annotated coding sequences in the curated mRNA database. In addition to the upstream ORFs in the presumed 5'-untranslated regions of mRNAs, bioinformatics analysis based on accumulated 5'-end cDNA sequence data provided evidence of novel short coding regions that were likely to be translated from the upstream non-AUG start site or from the new short transcript variants generated by utilization of downstream alternative promoters. Protein expression analysis of the *GRINL1A* gene revealed that translation from the most upstream start site occurred on the minor alternative splicing transcript, whereas this initiation site was not utilized on the major mRNA, resulting in translation of the downstream ORF from the second initiation codon. These findings reveal a novel post-transcriptional system that can augment the human proteome via the alternative use of diverse translation start sites coupled with transcriptional regulation through alternative promoters or splicing, leading to increased complexity of short protein-coding regions defined by the human transcriptome (Oyama et al., *Mol Cell Proteomics*, 6: 1000-1006, 2007).

7. Shotgun proteomics deciphered age/division of labor-related functional specification of three honeybee (*Apis mellifera* L.) exocrine glands

Toshiyuki Fujita⁵, Hiroko Kozuka-Hata, Yutaro Hori⁵, Jun Takeuchi⁵, Takeo Kubo⁵, and Masaaki Oyama: ⁵Department of Biological Sciences, Graduate School of Science, The University of Tokyo

The honeybee (*Apis mellifera* L.) uses various chemical signals produced by the worker exocrine glands to maintain the functioning of its colony. The roles of worker postcerebral glands (PcGs), thoracic glands (TGs), and mandibular glands (MGs) and the functional changes they undergo according to the division of labor from nursing to foraging are not as well studied. To comprehensively characterize the molecular roles of these glands in workers and their changes according to the division of labor of workers, we analyzed the proteomes of PcGs, TGs, and MGs from nurse bees and foragers using shotgun proteomics technology. We identified approximately 2000 proteins from each of the nurse bee or forager glands and highlighted the features of these glands at the molecular level by semiquantitative enrichment analyses of frequently detected, gland-selective, and labor-selective proteins. First, we found the high potential to produce lipids in PcGs and MGs, suggesting their relation to pheromone production. Second, we also found the proton pumps abundant in TGs and propose some transporters possibly related to the saliva production. Finally, our data unveiled candidate enzymes involved in labor-dependent acid production in MGs.

<Group II>

Biomolecular recognition is based on collective and specific non-covalent interactions between discrete biological molecules. Our laboratory studies a variety of protein systems, for instance antibody-antigen and receptor-ligand complexes, to understand quantitatively how these coordinated non-covalent interactions contribute to their specific recognition in biological and artificial systems. We seek to elucidate the molecular mechanisms by which biological molecules obtain high-specificity and affinity from multiple angles using advanced instrumentation. We aim to produce functional molecules with higher performance and better properties, to build a solid foundation from which to develop drugs that modulate specific interactions between biomolecules, and ultimately to understand the principles of molecular interactions in our lives.

1. Thermodynamic analyses of amino acid residues at the interface of an antibody B2212A and its antigen roundabout homolog 1

Yui A, Akiba H, Kudo S, Nakakido M, Nagatoishi S and Tsumoto K.

Artificial affinity maturation of antibodies is promising but often shows difficulties because the roles of each amino acid residue are not well known. To elucidate their roles in affinity against the antigen and thermal stability, interface residues in single-chain Fv of an antibody B2212A with its antigen roundabout homolog 1 were mutated and analyzed. Some amino acids played important roles in the affinity while others contributed to thermal stability.

2. Analysis of Pore Formation and Protein Translocation Using Large Biological Nanopores

Watanabe H, Gubbiotti A, Chinappi M, Takai N, Tanaka K, Tsumoto K and Kawano R.

This paper describes the analysis of pore formation and detection of a single protein molecule using a large nanopore among five different pore-forming proteins. We demonstrate that the identification of appropriate pores for nanopore sensing can be achieved by classifying the channel current signals and performing noise analysis. Through these analyses, we selected a perforin nanopore from the membrane attack complex/perforin superfamily and attempted to use it to detect the granzyme B protein, a serine protease. As a result, we found that granzyme B might pass through the perforin nanopore if it adopts an unfolded structure. Our proposed analytical approach should be useful for exploring several types of nanopore as large biological nanopores other than α -hemolysin.

3. Structure of the triose-phosphate/phosphate translocator reveals the basis of substrate specificity

Lee Y, Nishizawa T, Takemoto M, Kumazaki K, Yamashita K, Hirata K, Minoda A, Nagatoishi S, Tsumoto K, Ishitani R and Nureki O.

The triose-phosphate/phosphate translocator (TPT) catalyses the strict 1:1 exchange of triose-phosphate, 3-phosphoglycerate and inorganic phosphate across the chloroplast envelope, and plays crucial roles in photosynthesis. Despite rigorous study for more than 40 years, the molecular mechanism of TPT is poorly understood because of the lack of structural information. Here we report crystal structures of TPT bound to two different substrates, 3-phosphoglycerate and inorganic phosphate, in occluded conformations. The structures reveal that TPT adopts a 10-transmembrane drug/metabolite transporter fold. Both substrates are bound within the same central pocket, where conserved lysine, arginine and tyrosine residues recognize the shared phosphate group. A structural comparison with the outward-open conformation of the bacte-

rial drug/metabolite transporter suggests a rocker-switch motion of helix bundles, and molecular dynamics simulations support a model in which this rocker-switch motion is tightly coupled to the substrate binding, to ensure strict 1:1 exchange. These results reveal the unique mechanism of sugar phosphate/phosphate exchange by TPT.

4. Polymeric SpyCatcher Scaffold Enables Bioconjugation in a Ratio-Controllable Manner

Jia L, Minamihata K, Ichinose H, Tsumoto K and Kamiya N.

Conjugating enzymes into a large protein assembly often results in an enhancement of overall catalytic activity, especially when different types of enzymes that work cooperatively are assembled together. However, exploring the proper method to achieve protein assemblies with high stability and also to avoid loss of the function of each component for efficient enzyme clustering is remained challenging. Assembling proteins onto synthetic scaffolds through varied post-translational modification methods is particularly favored since the proteins can be site-specifically conjugated together with less activity loss. Here, a SpyCatcher polymer is prepared through catalytic reaction of horseradish peroxidase (HRP) and serves as a polymeric proteinaceous scaffold for construction of protein assemblies. Taking advantage of the favorable SpyCatcher-SpyTag interaction, SpyTagged proteins can be easily assembled onto the polymeric SpyCatcher scaffold with controllable binding ratio and site specificity. Firstly, the feasibility of construction of ratio-controllable binary artificial hemicellulosomes by assembling endoxylanase and arabinofuranosidase is explored. This construct achieves higher sugar conversion than that of the free enzymes when the proportion of arabinofuranosidase is high, because the close spatial proximity of the enzymes allows them to work in a synergistic manner. Another application for biosensing is developed by conjugating SpyTagged Nanoluc and protein G onto SpyCatcher polymer. Due to the protein clustering effect, an amplified luminescent intensity is achieved by the resulting conjugates than chimera protein of Nanoluc and protein G in ovalbumin detection in ELISA.

5. Biophysical characterization of the interaction between heme and proteins responsible for heme transfer in *Streptococcus pyogenes*

Hoshino M, Nakakido M, Nagatoishi S, Aikawa C, Nakagawa I and Tsumoto K.

Streptococcus pyogenes, an important pathogen that causes a wide range of diseases, possesses the

sia gene cluster, which encodes proteins involved in the heme acquisition system. Although this system was previously described, the molecular mechanism of effective heme transfer remains to be elucidated. Here, we have characterized the interactions between heme and each domain of Streptococcal hemoprotein receptor (Shr) and Streptococcal heme-binding protein (Shp). Our kinetic and thermodynamic analyses suggested that effective heme transfer within this system is achieved not only by affinity-based transfer but also by the difference of the binding driving force. The biophysical characterization of the above-mentioned interaction will lead to an indication for the selection of the target for a chemical screening of inhibitors as novel antibacterial agents based on biophysical approaches.

6. Structural basis for binding and transfer of heme in bacterial heme-acquisition systems

Naoe Y, Nakamura N, Rahman MM, Tosha T, Nagatoishi S, Tsumoto K, Shiro Y and Sugimoto H.

Periplasmic heme-binding proteins (PBPs) in Gram-negative bacteria are components of the heme acquisition system. These proteins shuttle heme across the periplasmic space from outer membrane receptors to ATP-binding cassette (ABC) heme importers located in the inner-membrane. In the present study, we characterized the structures of PBPs found in the pathogen *Burkholderia cenocepacia* (BhuT) and in the thermophile *Roseiflexus* sp. RS-1 (RhuT) in the heme-free and heme-bound forms. The conserved motif, in which a well-conserved Tyr interacts with the nearby Arg coordinates on heme iron, was observed in both PBPs. The heme was recognized by its surroundings in a variety of manners including hydrophobic interactions and hydrogen bonds, which was confirmed by isothermal titration calorimetry. Furthermore, this study of 3 forms of BhuT allowed the first structural comparison and showed that the heme-binding cleft of BhuT adopts an "open" state in the heme-free and 2-heme-bound forms, and a "closed" state in the one-heme-bound form with unique conformational changes. Such a conformational change might adjust the interaction of the heme(s) with the residues in PBP and facilitate the transfer of the heme into the translocation channel of the importer.

7. Elucidation of potential sites for antibody engineering by fluctuation editing

Yanaka S, Moriwaki Y, Tsumoto K and Sugase K.

Target-specific monoclonal antibodies can be routinely acquired, but the sequences of naturally acquired antibodies are not always affinity-matured and methods that increase antigen affinity are de-

sirable. Most biophysical studies have focused on the complementary determining region (CDR), which directly contacts the antigen; however, it remains difficult to increase the affinity as much as desired. While strategies to alter the CDR to increase antibody affinity are abundant, those that target non-CDR regions are scarce. Here we describe a new method, designated fluctuation editing, which identifies potential mutation sites and engineers a high-affinity antibody based on conformational fluctuations observed by NMR relaxation dispersion. Our data show that relaxation dispersion detects important fluctuating residues that are not located in the CDR and that increase antigen-antibody affinity by point mutation. The affinity-increased mutants are shown to fluctuate less in their free form and to form a more packed structure in their antigen-bound form.

8. Glycosylation of IgG-Fc: a molecular perspective

Kiyoshi M, Tsumoto K, Ishii-Watabe A and Caaveiro JMM.

Antibodies of the IgG class carry a pair of oligosaccharides (N-glycans) in the Fc region. The importance of the N-glycan is clearly demonstrated by its profound effect in the physicochemical and biological properties of antibodies. The term 'glycoengineering' has been coined to describe contemporary strategies to improve the performance of therapeutic monoclonal antibodies on the basis of modifications in the structure and composition of the N-glycan. These methodologies have resulted in the approval and commercialization of a new generation of antibodies with improved therapeutic efficacy. So far, these advances have been driven by herculean efforts in a process of trial-and-error. The collective work of researchers in this field is progressively revealing the molecular basis of N-glycans for the function of antibodies. This knowledge will ultimately be conducive to the application of rational approaches for the successful manipulation of antibodies using glycoengineering strategies. Herein, we review advances in our understanding of the role of the N-glycan in the structural and dynamic integrity, and biological activity, of antibodies. Since the N-glycan has a multifaceted effect in antibodies, in this review we have emphasized the importance of integrating various techniques that address this problem from multiple points of view. In particular, the combination of X-ray crystallography with nuclear magnetic resonance, molecular dynamics simulations and biophysical approaches based on thermodynamic principles, has emerged as a powerful combination that is deepening our understanding of this unique system with critical implications for human well-being.

9. Use of SpyTag/SpyCatcher to construct bispecific antibodies that target two epitopes of a single antigen

Yumura K, Akiba H, Nagatoishi S, Kusano-Arai O, Iwanari H, Hamakubo T and Tsumoto K.

Bispecific antibody targeting of two different antigens is promising, but when fragment-based antibodies are used, homogeneous production is difficult. To overcome this difficulty, we developed a method using the SpyTag/SpyCatcher system in which a covalent bond is formed between the two polypeptides. Using this method, we constructed a bispecific antibody that simultaneously interacted with two different epitopes of roundabout homologue 1 (ROBO1), a membrane protein associated with cancer progression. A bispecific tetravalent antibody with an additional functional moiety was also constructed by using a dimeric biotin-binding protein. An interaction analysis of ROBO1-expressing cells and the recombinant antigen demonstrated the improved binding ability of the bispecific antibodies through spontaneous binding of the two antibody fragments to their respective epitopes. In addition, multivalency delayed dissociation, which is advantageous in therapy and diagnosis.

10. Haemolytic actinoporins interact with carbohydrates using their lipid-binding module

Tanaka K, Caaveiro JMM, Morante K and Tsumoto K.

Pore-forming toxins (PFTs) are proteins endowed with metamorphic properties that enable them to stably fold in water solutions as well as in cellular membranes. PFTs produce lytic pores on the plasma membranes of target cells conducive to lesions, playing key roles in the defensive and offensive molecular systems of living organisms. Actinoporins are a family of potent haemolytic toxins produced by sea anemones vigorously studied as a paradigm of α -helical PFTs, in the context of lipid-protein interactions, and in connection with nanopore technologies. We have recently reported that fragaceatoxin C (FraC), an actinoporin, engages biological membranes with a large adhesive motif allowing the simultaneous attachment of up to four lipid molecules prior to pore formation. Since actinoporins also interact with carbohydrates, we sought to understand the molecular and energetic basis of glycan recognition by FraC. By employing structural and biophysical methodologies, we show that FraC engages glycans with low affinity using its lipid-binding module. Contrary to other PFTs requiring separate domains for glycan and lipid recognition, the small single-domain actinoporins economize resources by achieving dual recognition

with a single binding module. This mechanism could enhance the recruitment of actinoporins to the surface of target tissues in their marine environment. This article is part of the themed issue 'Membrane pores: from structure and assembly, to medicine and technology'.

11. Functional Contacts between MPER and the Anti-HIV-1 Broadly Neutralizing Antibody 4E10 Extend into the Core of the Membrane

Rujas E, Insausti S, García-Porras M, Sánchez-Eugenia R, Tsumoto K, Nieva JL and Caaveiro JM.

The exceptional breadth of broadly neutralizing antibodies (bNAbs) against the membrane-proximal external region (MPER) of the transmembrane protein gp41 makes this class of antibodies an ideal model to design HIV vaccines. From a practical point of view, however, the preparation of vaccines eliciting bNAbs is still a major roadblock that limits their clinical application. Fresh mechanistic insights are necessary to develop more effective strategies. In particular, the function of the unusually long complementarity-determining region three of the heavy chain (CDRH3) of 4E10, an anti-MPER bNAb, is an open question that fascinates researchers in the field. Residues comprising the apex region are dispensable for engagement of the epitope in solution; still, their single mutation profoundly impairs the neutralization capabilities of the antibody. Since this region is very hydrophobic, it has been proposed that the apex is essential for anchoring the antibody to the viral membrane where MPER resides. Herein, we have critically examined this idea using structural, biophysical, biochemical, and cell-based approaches. Our results demonstrate that the apex region is not just a "greasy" spot merely increasing the affinity of the antibody for the membrane. We demonstrate the three-dimensional engagement of the apex region of the CDRH3 with the conglomerate of gp41 epitope and membrane lipids as a means of effective binding and neutralization of the virus. This mechanism of recognition suggests a standard route of antibody ontogeny. Therefore, we need to focus our efforts on recreating a more realistic MPER/lipid immunogen in order to generate more effective anti-HIV-1 vaccines.

12. Peripheral Membrane Interactions Boost the Engagement by an Anti-HIV-1 Broadly Neutralizing Antibody

Rujas E, Caaveiro JM, Insausti S, García-Porras M, Tsumoto K and Nieva JL.

The 4E10 antibody displays an extreme breadth

of HIV-1 neutralization and therefore constitutes a suitable model system for structure-guided vaccine design and immunotherapeutics against AIDS. In this regard, the relevance of autoreactivity with membrane lipids for the biological function of this antibody is still a subject of controversy. To address this dispute, herein we have compared the membrane partitioning ability of the 4E10 antibody and several of its variants, which were mutated at the region of the paratope surface in contact with the membrane interface. We first employed a physical separation approach (vesicle flotation) and subsequently carried out quantitative fluorescence measurements in an intact system (spectroscopic titration), using 4E10 Fab labeled with a polarity-sensitive fluorescent probe. Moreover, recognition of epitope peptide in membrane was demonstrated by photo-cross-linking assays using a Fab that incorporated the genetically encoded unnatural amino acid *p*-benzoylphenylalanine. The experimental data ruled out that the proposed stereospecific recognition of viral lipids was necessary for the function of the antibody. In contrast, our data suggest that nonspecific electrostatic interactions between basic residues of 4E10 and acidic phospholipids in the membranes contribute to the observed biological function. Moreover, the energetics of membrane partitioning indicated that 4E10 behaves as a peripheral membrane protein, tightening the binding to the ligand epitope inserted in the viral membrane. The implications of these findings for the natural production and biological function of this antibody are discussed.

13. The carboxyl-terminal region of Dok-7 plays a key, but not essential, role in activation of muscle-specific receptor kinase MuSK and neuromuscular synapse formation

Ueta R, Tezuka T, Izawa Y, Miyoshi S, Nagatoishi S, Tsumoto K and Yamanashi Y.

As the synapse between a motor neuron and skeletal muscle, the neuromuscular junction (NMJ) is required for muscle contraction. The formation and maintenance of NMJs are controlled by the muscle-specific receptor kinase MuSK. Dok-7 is the essential cytoplasmic activator of MuSK, and indeed mice lacking Dok-7 form no NMJs. Moreover, DOK7 gene mutations underlie DOK7 myasthenia, an NMJ synaptopathy. Previously, we failed to detect MuSK activation in myotubes by Dok-7 mutated in the N-terminal pleckstrin homology (PH) or phosphotyrosine binding (PTB) domain or that lacked the C-terminal region (Dok-7- Δ C). Here, we found by quantitative analysis that Dok-7- Δ C marginally, but significantly, activated MuSK in myotubes, unlike the PH- or PTB-mutant. Purified, recombinant Dok-7- Δ C, but not other mutants, also

showed marginal ability to activate MuSK's cytoplasmic portion, carrying the kinase domain. Consistently, forced expression of Dok-7- Δ C rescued Dok-7-deficient mice from neonatal lethality caused by the lack of NMJs, indicating restored MuSK activation and NMJ formation. However, these mice showed only marginal activation of MuSK and died by 3 weeks of age apparently due to an abnormally small number and size of NMJs. Thus, Dok-7's C-terminal region plays a key, but not fully essential, role in MuSK activation and NMJ formation.

14. Disruption of cell adhesion by an antibody targeting the cell-adhesive intermediate (X-dimer) of human P-cadherin

Kudo S, Caaveiro JM, Nagatoishi S, Miyafusa T, Matsuura T, Sudou Y and Tsumoto K.

Human P-cadherin is a cell adhesion protein of the family of classical cadherins, the overexpression of which is correlated with poor prognosis in various types of cancer. Antibodies inhibiting cell-cell adhesion mediated by P-cadherin show clear therapeutic effect, although the mechanistic basis explaining their effectiveness is still unclear. Based on structural, physicochemical, and functional analyses, we have elucidated the molecular mechanism of disruption of cell adhesion by antibodies targeting human P-cadherin. Herein we have studied three different antibodies, TSP5, TSP7, and TSP11, each recognizing a different epitope on the surface of the cell-adhesive domain (EC1). Although all these three antibodies recognized human P-cadherin with high affinity, only TSP7 disrupted cell adhesion. Notably, we demonstrated that TSP7 abolishes cell adhesion by disabling the so-called X-dimer (a kinetic adhesive intermediate), in addition to disrupting the strand-swap dimer (the final thermodynamic state). The inhibition of the X-dimer was crucial for the overall inhibitory effect, raising the therapeutic value of a kinetic intermediary not only for preventing, but also for reversing, cell adhesion mediated by a member of the classical cadherin family. These findings should help to design more innovative and effective therapeutic solutions targeting human P-cadherin.

15. Through-bond effects in the ternary complexes of thrombin sandwiched by two DNA aptamers

Pica A, Russo Krauss I, Parente V, Tateishi-Karimata H, Nagatoishi S, Tsumoto K, Sugimoto N and Sica F.

Aptamers directed against human thrombin can selectively bind to two different exosites on the protein surface. The simultaneous use of two DNA

aptamers, HD1 and HD22, directed to exosite I and exosite II respectively, is a very powerful approach to exploit their combined affinity. Indeed, strategies to link HD1 and HD22 together have been proposed in order to create a single bivalent molecule with an enhanced ability to control thrombin activity. In this work, the crystal structures of two ternary complexes, in which thrombin is sandwiched between two DNA aptamers, are presented and discussed. The structures shed light on the cross talk between the two exosites. The through-bond effects are particularly evident at exosite II, with net consequences on the HD22 structure. Moreover, thermodynamic data on the binding of the two aptamers are also reported and analyzed.

16. Production and characterization of genetically modified human IL-11 variants

Sano E, Takei T, Ueda T and Tsumoto K.

Interleukin-11 (IL-11) has been expected as a drug on severe thrombocytopenia caused by myelosuppressive chemotherapy. Whereas, development of IL-11 inhibitor is also expected for a treatment against IL-11 related cancer progression. Here, we will demonstrate the creation of various kinds of genetically modified hIL-11s. Modified vectors were constructed by introducing N- or O-glycosylation site on the region of hIL-11 that does not belong to the core α -helical motif based on the predicted secondary structure. N-terminal (N: between 22 to 23 aa), the first loop (M1:70 to 71 aa), the second loop (M2:114-115 aa), the third loop (M3:160-161 aa) and C-terminal (C: 200- aa) were selected for modification. A large scale production system was established and the characteristics of modified hIL-11s were evaluated. The structure was analyzed by amino acid sequence and composition analysis and CD-spectra. Glycan was assessed by monosaccharide composition analysis. Growth promoting activity and biological stability were analyzed by proliferation of T1165 cells. N-terminal modified proteins were well glycosylated and produced. Growth activity of 3NN with NASNASNAS sequence on N-terminal was about tenfold higher than wild type (WT). Structural and biological stabilities of 3NN were also better than WT and residence time in mouse blood was longer than WT. M1 variants lacked growth activity though they are well glycosylated and secondary structure is very stable. Both of 3NN and OM1 with AAATPAPG on M1 associated with hIL-11R strongly. These results indicate N-terminal and M1 variants will be expected for practical use as potent agonists or antagonists of hIL-11.

17. Improved Brain Expression of Anti-Amyloid β scFv by Complexation of mRNA Including a Secretion Sequence with PEG-based Block Catiomer

Perche F, Uchida S, Akiba H, Lin CY, Ikegami M, Dirisala A, Nakashima T, Itaka K, Tsumoto K and Kataoka K.

The ever-increasing number of people living with Alzheimer's disease urges to develop more effective therapies. Despite considerable success, anti-Alzheimer immunotherapy still faces the challenge of intracerebral and intracellular delivery. This work introduces in situ production of anti-amyloid beta ($A\beta$) antibody after intracerebral injection of PEG-PAsp(DET)/mRNA polyplexes as a novel immunotherapy approach and a safer alternative compared to high systemic antibodies doses or administration of adenovirus encoding anti- $A\beta$ antibodies.

<Group III>

1. Development of new methods for analyzing neural circuits in the retina

Neural circuits in the central nervous system are the basis of various high-order brain functions. It is also true in case of retina. In the retina, six main classes of neural cells connect systematically to make up complex neural circuits. Characteristics of the retinal neural cell functions have been examined mainly by the electrophysiological methods and models of cell connectivity have been proposed. Morphological studies of the actual neural connection, which constitute the physiological properties of retinal neurons, have been desired. Until recently the only method to reveal the connectivity of actual neural cells morphologically was to collect ultrathin serial sections and observe in transmission electron microscope (TEM). But the technical difficulties discouraged us from such a troublesome studies. Recent progress in scanning electron microscope (SEM) equipment allowed us to develop a new method to observe ultrathin TEM sections in SEM. To observe thin TEM sections, we have developed new sample staining methods to enhance electron contrast. To collect huge number of serial sections stably and efficiently, we have been developing new equipment and techniques. By using this equipment, it became possible to collect more than 1000 serial sections of less than 30nm thickness much easier. We have analyzed about 500 serial thin sections of zebrafish retinal outer plexiform layer by this method and succeeded in tracing thin processes of bipolar cells into the photoreceptor terminal. By using SEM to observe thin TEM sections, it became possible to analyze much wider areas than by using TEM. These methods are also appli-

cable for analyzing other cells and tissues and such studies are currently in progress.

2. Collaborative and supportive works as electron microscope core-laboratory

This group is also engaged in collaborative researches using electron microscope. We offer supports for the research projects those need electron microscopic analysis. The services available in this group are the conventional thin section transmission electron microscopy, immuno-electron microscopy, negative staining techniques and scanning electron microscopy. By using these individual technique or combination of some of these we can offer direct visual evidence that cannot be acquired by other methods. This year, 18 projects in 13 laboratories were performed as core-laboratory works.

a. Thin section transmission electron microscopy

Thin section transmission electron microscopy is the most widely used technique to observe the inner structure of cells and tissues. In this method, samples are fixed and embedded in epoxy resin, thin sections with about 70nm thickness are cut and observed in the electron microscope. In case of immuno-electron microscopy, thin sections are obtained by similar procedure, and the antigen epitopes exposed on the surface of the sections are marked by sequentially reacted with appropriate primary antibodies and colloidal gold labeled secondary antibodies. This year, thin section electron microscopy combined with immuno-electron microscopy were used in many collaborative works.

a-1. Ultrastructural analysis of entry and assembly of Herpes Simplex Virus

We have been performing several studies with research groups in Dr. Kawaguchi's laboratory: ¹Division of Molecular Virology, Department of Microbiology and Immunology, regarding the infection/replication processes of herpes simplex virus (HSV). Thin section electron microscopy have been used to analyze the function of viral proteins in trans-nuclear membrane processes of the newly formed viruses. By analyzing the virus forming processes in some mutant host cells, we could analyze viral proteins as well as candidate host molecules those may be involved in the trans-nuclear process of the HSV. This year, we also used serial ultra thin section analysis for correlative light and electron microscopy (CLEM) analysis and demonstrated that the accumulations of viral protein detected in confocal light microscopy correspond to the viral capsid accumulations (ref. Kobayashi R.¹ *et al*).

a-2. Essential role of docosahexaenoic acid in visual function by formation of photoreceptor cells

We have been performing several studies also with research groups in Dr. Watanabe²'s laboratory: ²Project Division of Molecular and Developmental Biology. This year, we evaluated the need of docosahexaenoic acid (DHA) in retinal development. With the analysis of the retina by electron microscopy, we estimated that DHA-containing phospholipids might be more essential for disc morphogenesis and/or maintenance than for new disc formation at the basal OS (ref. Shindou² *et al.*).

Some other collaborative research works using thin section electron microscopy and/or immunoelectron microscopy were performed with Dr. Kamioka³, ³Division of Mucosal Immunology, concerning function of intestinal Paneth cells in mucosal immunity, Dr. Shibata⁴, in ⁴Division of Infectious Genetics, Dr. Takekawa⁵ in ⁵Division of Cell Signaling and Molecular Medicine and so on.

b. Negative staining techniques

Negative staining techniques are simple and quick method to observe the morphology of the macro-molecules. This year, negative staining techniques combined with scanning electron microscopy were used to analyze the function of a protein during in vitro formation of collagen fibers in collaboration with Dr. Tashima⁶ *et al.* in ⁶Medical Proteomics Laboratory.

c. Conventional scanning electron microscopy

Conventional scanning electron microscopy is a technique used to examine the surface structure of the cells, tissues or other non-biological materials. The collaborative works using scanning electron microscopy were done with Dr. Mimuro⁷'s group, in ⁷Division of Bacteriology, Department of Infectious Disease Control, International Research Center for Infectious Diseases, about the morphological conversions of *Helicobacter pylori* under anaerobic conditions. Scanning electron microscopy was also used to analyze the morphological changes of collagen fibers as a collaborative work with Tashima⁶ *et al.*

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