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. Proteome-wide analysis of lysine acetylation and ubiquitination reveals critical signaling regulation in cancer cells

Hiroko Kozuka-Hata, Aya Kitamura, Tomoko Hiroki, Aiko Aizawa, Kouhei Tsumoto, Jun-ichiro Inoue and Masaaki Oyama.

Post-translational modifications (PTMs), such as phosphorylation, ubiquitination and acetylation, are known to be widely involved in the regulation of various biological processes through extensive diversification of each protein function at the cellular network level. Previous functional analyses of cancer cell signaling under a variety of experimental conditions revealed many of the key molecules and their associated protein modifications in relation to each type of cancer. In order to systematically discover critical modulators from diversified signaling molecules, we have developed a high-resolution mass spectrometry-based proteomics platform for integrative identification and quantification of multiple post-translational modifications from various types of cancer cells. Our large-scale proteomic analysis enabled us to identify more than 5,000 kinds of ubiquitinated sites and 1,600 kinds of acetylated sites from representative human cancer cell lines, leading to identification of approximately 900 novel lysine modification sites in total. Very interestingly, 236 lysine residues derived from 141 proteins were found to be modified with both ubiquitination and acetylation. As a consequence of the subsequent motif extraction analyses, glutamic acid (E) was found to be highly enriched at the position (-1) for the lysine acetylation sites, whereas the same amino acid was relatively dispersed along the neighboring residues of the lysine ubiquitination sites.

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

Hiroko Kozuka-Hata, Tomoko Hiroki, Ryo Koyama-Nasu[1], Kouhei Tsumoto, Jun-ichiro Inoue, Tetsu Akiyama[1] and Masaaki Oyama; [1]Laboratory of Molecular and Genetic Information, Institute for Quantitative Biosciences, The University of Tokyo

As glioblastoma is the most common and aggres-

sive 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 (TGFBR2) as a Novel Regulator of Glioblastoma Stem Cell Properties

Yuta Narushima, Hiroko Kozuka-Hata, Ryo Koyama-Nasu[1], Kouhei Tsumoto, Jun-ichiro Inoue, Tetsu Akiyama[1] 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 (TGFBR2) 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

Hiroko Kozuka-Hata, Masato Suzuki[2], Kotaro Kiga[2], Shinya Tasaki, Jun-ichiro Inoue, Tadashi Yamamoto[3], Chihiro Sasakawa[2] and Masaaki Oyama: [2]Division of Bacterial Infection, Department of Microbiology and Immunology, IMSUT, [3]Division of Oncology, Department of Cancer Biology, IMSUT

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 Fujita[5], Hiroko Kozuka-Hata, Yutaro Hori[5], Jun Takeuchi[5], Takeo Kubo[5], and Masaaki Oyama: [5]Department 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, such as antibody-antigen and protein-ligand complexes, to understand quantitatively how these coordinated non-covalent interactions contribute to the specific recognition in biological and artificial systems. We seek to elucidate the molecular mechanisms by which biological molecules generate high specificity and affinity from multiple angles using advanced instruments. We aim to produce functional molecules with higher performance and better properties and to build solid foundations to develop drugs that modulate specific interactions between biomolecules, helping to understand the principles of molecular interactions in our lives.

1. Effects of a remote mutation from the contact paratope on the structure of CDR-H3 in the anti-HIV neutralizing antibody PG16

Kondo HX, Kiribayashi R, Kuroda D, Kohda J, Kugimiya A, Nakano Y, Tsumoto K, and Takano Y.

PG16 is a broadly neutralizing antibody to the human immunodeficiency virus (HIV). A crystal structure of PG16 revealed that the unusually long 28-residue complementarity determining region (CDR) H3 forms a unique subdomain, referred to as a "hammerhead", that directly contacts the antigen. The hammerhead apparently governs the function of PG16 while a previous experimental assay showed that the mutation of Tyr^{H100Q} to Ala, which does not directly contact the antigen, decreased the neutralization ability of PG16. However, the molecular mechanism by which a remote mutation from the hammerhead or contact paratope affects the neutralization potency has remained unclear. Here, we performed molecular dynamics simulations of the wild-type and variants (Tyr^{H100Q} to Ala, and Tyr^{H100Q} to Phe) of PG16, to clarify the effects of these mutations on the dynamics of CDR-H3. Our simulations revealed that the structural rigidity of the CDR-H3 in PG16 is attributable to the hydrogen bond interaction between Tyr^{H100Q} and Pro^{H99}, as well as the steric support by Tyr^{H100Q}. The loss of both interactions increases the intrinsic fluctuations of the CDR-H3 in PG16, leading to a conformational transition of CDR-H3 toward an inactive state.

2. How the protonation state of a phosphorylated amino acid governs molecular recognition: insights from classical molecular dynamics simulations

Kawade R, Kuroda D, and Tsumoto K.

Physicochemical properties of proteins are controlled mainly by post-translational modifications such as amino acid phosphorylation. Although molecular dynamics simulations have been shown to be a valuable tool for studying the effects of phosphorylation on protein structure and dynamics, most of the previous studies assumed that the phosphate group was in the unprotonated (PO2−3) state, even though the protonation state could in fact vary at physiological pH. In this study, we performed molecular dynamics simulations of four different protein-phosphorylated peptide complexes both in the PO2−3 and PO3 H- states. Our simulations delineate different dynamics and energetics between the two states, suggesting importance of the protonation state of a phosphorylated amino acid in molecular recognition.

3. Technical Capabilities and Limitations of Optical Spectroscopy and Calorimetry Using Water-Miscible Solvents: The Case of Dimethyl Sulfoxide, Acetonitrile, and 1,4-Dioxane

Hirano A, Nagatoishi S, Wada M, Tsumoto K, Maluf KN, and Arakawa T.

In drug development, water-miscible solvents are commonly used to dissolve drug substances. Typical routine procedures in drug development include dilution of the stock drug solution into an aqueous solution containing target macromolecules for drug binding assays. However, water-miscible solvents impose some technical limitations on the assays on account of their light absorption and heat capacity. Here, we examined the effects of the dilution of 3 water-miscible solvents, that is, dimethyl sulfoxide, acetonitrile, and 1,4-dioxane, on the baseline stability and signal/noise ratio in circular dichroism spectroscopy, isothermal titration calorimetry, and differential scanning calorimetry. Dimethyl sulfoxide and 1,4-dioxane affect the signal/noise ratio of circular dichroism spectra at typically used concentrations due to their light absorbance. The water-miscible solvents generate interfering signals in the isothermal titration calorimetry due to their mixing heat. They show negative or positive slope in the differential scanning calorimetry.

Such interfering effects of the solvents are reduced by appropriate dilution according to the analytical techniques. Because the water-miscible solvents have solubilization capacity for alkyl chain moieties and aromatic moieties of chemicals, drug substances containing these moieties can be dissolved into the solvents and then subjected to the analyses to examine their interactions with target proteins after appropriate dilution of the drug solutions.

4. Structural and thermodynamic basis for the recognition of the substrate-binding cleft on hen egg lysozyme by a single-domain antibody

Akiba H, Tamura H, Kiyoshi M, Yanaka S, Sugase K, Caaveiro JMM, and Tsumoto K.

Single-domain antibodies (VHHs or nanobodies), developed from heavy chain-only antibodies of camelids, are gaining attention as next-generation therapeutic agents. Despite their small size, the high affinity and specificity displayed by VHHs for antigen molecules rival those of IgGs. How such small antibodies achieve that level of performance? Structural studies have revealed that VHHs tend to recognize concave surfaces of their antigens with high shape-complementarity. However, the energetic contribution of individual residues located at the binding interface has not been addressed in detail, obscuring the actual mechanism by which VHHs target the concave surfaces of proteins. Herein, we show that a VHH specific for hen egg lysozyme, D3-L11, not only displayed the characteristic binding of VHHs to a concave region of the surface of the antigen, but also exhibited a distribution of energetic hot-spots like those of IgGs and conventional protein-protein complexes. The highly preorganized and energetically compact interface of D3-L11 recognizes the concave epitope with high shape complementarity by the classical lock-and-key mechanism. Our results shed light on the fundamental basis by which a particular VHH accommodate to the concave surface of an antigens with high affinity in a specific manner, enriching the mechanistic landscape of VHHs.

5. ^{111}In-labeled anti-cadherin17 antibody D2101 has potential as a noninvasive imaging probe for diagnosing gastric cancer and lymph-node metastasis

Fujiwara K, Tsuji AB, Sudo H, Sugyo A, Akiba H, Iwanari H, Kusano-Arai O, Tsumoto K, Momose T, Hamakubo T, and Higashi T.

Cadherin-17 (CDH17) is a transmembrane protein that mediates cell-cell adhesion and is frequently expressed in adenocarcinomas, including gastric cancer. CDH17 may be an effective diagnostic marker for the staging of gastric cancer. We developed an 111In-labeled anti-CDH17 monoclonal antibody (Mab) as an imaging tracer and performed biodistribution and single-photon emission computed tomography (SPECT)/computed tomography (CT) imaging studies using mice with CDH17-positive gastric cancer xenografts. CDH17 expression in gastric cancer specimens was also analyzed.

Surface plasmon resonance analysis revealed that D2101 specifically recognizes human CDH17, but not murine CDH17. The affinity of D2101 slightly decreased as a result of the radiolabeling procedures. The biodistribution study revealed high uptake of 111In-D2101 in tumors (maximum, 39.2 ± 9.5% ID/g at 96 h postinjection), but low uptake in normal organs, including the stomach. Temporal SPECT/CT imaging with 111In-D2101 visualized tumors with a high degree of tumor-to-nontumor contrast. Immunohistochemical analysis revealed that, compared with HER2, which is a potential marker of N-stage, CDH17 had a higher frequency of positivity in specimens of primary and metastatic gastric cancer. Our 111In-anti-CDH17 Mab D2101 depicted CDH17-positive gastric cancer xenografts in vivo and has the potential to be an imaging probe for the diagnosis of primary lesions and lymph-node metastasis in gastric cancer.

6. Biophysical characterization of the breast cancer-related BIG3-PHB2 interaction: Effect of non-conserved loop region of BIG3 on the structure and the interaction

Chigira T, Nagatoishi S, Takeda H, Yoshimaru T, Katagiri T, and Tsumoto K.

Brefeldin A-inhibited guanine nucleotide-exchange protein 3 (BIG3) interacts with and inhibits the tumor suppressor function of prohibitin-2 (PHB2), and recent in vivo studies have demonstrated that the BIG3-PHB2 interaction is a promising target for breast cancer therapy. However, little biophysical characterization on BIG3 and its interaction with PHB2 has been reported. Here we compared the calculated 8-class secondary structure of the N-terminal domains of BIG family proteins and identified a loop region unique to BIG3. Our biophysical characterization demonstrated that this loop region significantly affects the colloidal and thermodynamic stability of BIG3 and the thermodynamic and kinetic profile of its interaction with PHB2. These results establish a model for the BIG3-PHB2 interaction and an entry for drug discovery for breast cancer.

7. A Peptoid with Extended Shape in Water

Morimoto J, Fukuda Y, Kuroda D, Watanabe T, Yoshida F, Asada M, Nakamura T, Senoo A, Nagatoishi S, Tsumoto K, and Sando S.

The term "peptoids" was introduced decades ago to describe peptide analogues that exhibit better physicochemical and pharmacokinetic properties than peptides. Oligo(N-substituted glycine) (oligo-NSG) was previously proposed as a peptoid due to its high proteolytic resistance and membrane permeability. However, oligo-NSG is conformationally flexible, and ensuring a defined shape in water is difficult. This conformational flexibility severely limits the biological application of oligo-NSG. Here, we propose oligo(N-substituted alanine) (oligo-NSA) as a peptoid that forms a defined shape in water. The synthetic method established in this study enabled the first isolation and conformational study of optically pure oligo-NSA. Computational simulations, crystallographic studies, and spectroscopic analysis demonstrated the well-defined extended shape of oligo-NSA realized by backbone steric effects. This new class of peptoid achieves the constrained conformation without any assistance of N-substituents and serves as a scaffold for displaying functional groups in well-defined three-dimensional space in water, which leads to effective biomolecular recognition.

8. Highly sensitive biomolecular interaction detection method using optical bound/free separation with grating-coupled surface plasmon field-enhanced fluorescence spectroscopy (GC-SPFS)

Kaya T, Nagatoishi S, Nagae K, Nakamura Y, and Tsumoto K.

Grating-coupled surface plasmon field-enhanced fluorescence spectroscopy (GC-SPFS) with optical bound/free (B/F) separation technique was developed by employing a highly directional fluorescence with polarization of surface plasmon-coupled emission (SPCE) to realize highly sensitive immunoassay regardless of the ligand affinity. A highly sensitive immunoassay system with GC-SPFS was constructed using a plastic sensor chip reproducibly fabricated in-house by nanoimprinting and applied to the quantitative detection of an anti-lysozyme single-domain antibody (sdAb), to compare conventional washing B/F separation with optical B/F separation. Differences in the affinity of the anti-lysozyme sdAb, induced by artificial mutation of only one amino acid residue in the variable domain were attributed to higher sensitivity than that of the conventional Biacore surface plasmon resonance (SPR) system. The detection limit (LOD; means of six replicates of the zero standard plus three standard deviations) of the GC-SPFS immunoassay with optical B/F separation, was estimated to be 1.2 ng/ml with the low-affinity ligand (mutant sdAb Y52A: KD level was of the order of 10-7 ~ 10-6 M) and was clearly improved as compared to that (LOD: 9.4 ng/ml) obtained with the conventional washing B/F separation. These results indicate that GC-SPFS with the optical B/F separation technique offers opportunities to re-evaluate low-affinity biomaterials that are neither fully utilized nor widespread, and could facilitate the creation of novel and innovative methods in drug and diagnostic development.

9. The Isolation of New Pore-Forming Toxins from the Sea Anemone Actinia fragacea Provides Insights into the Mechanisms of Actinoporin Evolution

Morante K, Bellomio A, Viguera AR, González-Mañas JM, Tsumoto K, and Caaveiro JMM.

Random mutations and selective pressure drive protein adaptation to the changing demands of the environment. As a consequence, nature favors the evolution of protein diversity. A group of proteins subject to exceptional environmental stress and known for their widespread diversity are the pore-forming hemolytic proteins from sea anemones, known as actinoporins. In this study, we identified and isolated new isoforms of actinoporins from the sea anemone Actinia fragacea (fragaceatoxins). We characterized their hemolytic activity, examined their stability and structure, and performed a comparative analysis of their primary sequence. Sequence alignment reveals that most of the variability among actinoporins is associated with non-functional residues. The differences in the thermal behavior among fragaceatoxins suggest that these variability sites contribute to changes in protein stability. In addition, the protein-protein interaction region showed a very high degree of identity (92%) within fragaceatoxins, but only 25% among all actinoporins examined, suggesting some degree of specificity at the species level. Our findings support the mechanism of evolutionary adaptation in actinoporins and reflect common pathways conducive to protein variability.

10. Structural features of methionine aminopeptidase2-active core peptide essential for binding with S100A4

Katagiri N, Nagatoishi S, Tsumoto K, and Endo H.

Methionine aminopeptidase 2 (MetAP2) is one of the effector proteins of S100A4, a metastasis-associated calcium-binding protein. This interaction is involved in angiogenesis. The region of MetAP2 that interacts with S100A4 includes amino acids 170 to 208. A peptide corresponding to this region, named as NBD, has potent anti-angiogenic activity and suppresses tumor growth in a xenograft cancer model. However, the binding mode of NBD to S100A4 was totally unknown. Here we describe our analysis of the relationship between the inhibitory activity and the structure of NBD, which adopts a characteristic helix-turn-helix structure as shown by X-ray crystallo-

graphic analysis, and peptide fragments of NBD. We conducted physicochemical analyses of the interaction between S100A4 and the peptides, including surface plasmon resonance, microscale thermophoresis, and circular dichroism, and performed docking/molecular dynamics simulations. Active peptides had stable secondary structures, whereas inactive peptides had a little secondary structure. A computational analysis of the interaction mechanism led to the design of a peptide smaller than NBD, NBD-ΔN10, that possessed inhibitory activity. Our study provides a strategy for design for a specific peptide inhibitor against S100A4 that can be applied to the discovery of inhibitors of other protein-protein interactions.

11. Identification of key modules and hub genes for small-cell lung carcinoma and large-cell neuroendocrine lung carcinoma by weighted gene co-expression network analysis of clinical tissue-proteomes

Nakamura H, Fujii K, Gupta V, Hata H, Koizumu H, Hoshikawa M, Naruki S, Miyata Y, Takahashi I, Miyazawa T, Sakai H, Tsumoto K, Takagi M, Saji H, and Nishimura T.

Small-cell lung carcinoma (SCLC) and large-cell neuroendocrine lung carcinoma (LCNEC) are high-grade lung neuroendocrine tumors (NET). However, comparative protein expression within SCLC and LCNEC remains unclear. Here, protein expression profiles were obtained via mass spectrometry-based proteomic analysis. Weighted gene co-expression network analysis (WGCNA) identified co-expressed modules and hub genes. Of 34 identified modules, six were significant and selected for protein-protein interaction (PPI) network analysis and pathway enrichment. Within the six modules, the activation of cellular processes and complexes, such as alternative mRNA splicing, translation initiation, nucleosome remodeling and deacetylase (NuRD) complex, SWItch/Sucrose Non-Fermentable (SWI/SNF) superfamily-type complex, chromatin remodeling pathway, and mRNA metabolic processes, were significant to SCLC. Modules enriched in processes, including signal recognition particle (SRP)-dependent co-translational protein targeting to membrane, nuclear-transcribed mRNA catabolic process of nonsense-mediated decay (NMD), and cellular macromolecule catabolic process, were characteristically activated in LCNEC. Novel high-degree hub genes were identified for each module. Master and upstream regulators were predicted via causal network analysis. This study provides an understanding of the molecular differences in tumorigenesis and malignancy between SCLC and LCNEC and may help identify potential therapeutic targets.

12. An epitope-directed antibody affinity maturation system utilizing mammalian cell survival as readout

Eguchi A, Nakakido M, Nagatoishi S, Kuroda D, Tsumoto K, Nagamune T, and Kawahara M.

Upon developing therapeutically potent antibodies, there are significant requirements, such as increasing their affinity, regulating their epitope, and using native target antigens. Many antibody selection systems, such as a phage display method, have been developed, but it is still difficult to fulfill these requirements at the same time. Here, we propose a novel epitope-directed antibody affinity maturation system utilizing mammalian cell survival as readout. This system is based on the competition of antibody binding, and can target membrane proteins expressed in a native form on a mammalian cell surface. Using this system, we successfully selected an affinity-matured anti-ErbB2 single-chain variable fragment variant, which had the same epitope as the original one. In addition, the affinity was increased mainly due to the decrease in the dissociation rate. This novel cell-based antibody affinity maturation system could contribute to directly obtaining therapeutically potent antibodies that are functional on the cell surface.

13. Exploring designability of electrostatic complementarity at an antigen-antibody interface directed by mutagenesis, biophysical analysis, and molecular dynamics simulations

Yoshida K, Kuroda D, Kiyoshi M, Nakakido M, Nagatoishi S, Soga S, Shirai H, and Tsumoto K.

Antibodies protect organisms from a huge variety of foreign antigens. Antibody diversity originates from both genetic and structural levels. Antigen recognition relies on complementarity between antigen-antibody interfaces. Recent methodological advances in structural biology and the accompanying rapid increase of the number of crystal structures of proteins have enabled atomic-level manipulation of protein structures to effect alterations in function. In this study, we explored the designability of electrostatic complementarity at an antigen-antibody interface on the basis of a crystal structure of the complex. We designed several variants with altered charged residues at the interface and characterized the designed variants by surface plasmon resonance, circular dichroism, differential scanning calorimetry, and molecular dynamics simulations. Both successes and failures of the structure-based design are discussed. The variants that compensate electrostatic interactions can restore the interface complementarity, enabling the cognate antigen-antibody binding. Retrospectively, we also show that these mutational effects could be predicted by the simulations. Our study

demonstrates the importance of charged residues on the physical properties of this antigen-antibody interaction and suggests that computational approaches can facilitate design of antibodies that recognize a weakly immunogenic antigen.

14. Design strategy for serine hydroxymethyltransferase probes based on retro-aldol-type reaction

Nonaka H, Nakanishi Y, Kuno S, Ota T, Mochidome K, Saito Y, Sugihara F, Takakusagi Y, Aoki I, Nagatoishi S, Tsumoto K, and Sando S.

Serine hydroxymethyltransferase (SHMT) is an enzyme that catalyzes the reaction that converts serine to glycine. It plays an important role in one-carbon metabolism. Recently, SHMT has been shown to be associated with various diseases. Therefore, SHMT has attracted attention as a biomarker and drug target. However, the development of molecular probes responsive to SHMT has not yet been realized. This is because SHMT catalyzes an essential yet simple reaction; thus, the substrates that can be accepted into the active site of SHMT are limited. Here, we focus on the SHMT-catalyzed retro-aldol reaction rather than the canonical serine-glycine conversion and succeed in developing fluorescent and 19F NMR molecular probes. Taking advantage of the facile and direct detection of SHMT, the developed fluorescent probe is used in the high-throughput screening for human SHMT inhibitors, and two hit compounds are obtained.

15. Control of Protein Adsorption to Cyclo Olefin Polymer by the Hofmeister Effect

Fujita R, Nagatoishi S, Adachi S, Nishioka H, Ninomiya H, Kaya T, Takai M, Arakawa T, and Tsumoto K.

Cyclo olefin polymer (COP) is an attractive plastic because it has low protein adsorption despite its hydrophobic chemical structure. Here, the adsorption of model proteins to the COP was evaluated in comparison with a representative plastic, polystyrene (PSt), using reflectometry interference spectroscopy (RIfS) technology. The effects of different salts on adsorption were then examined. The adsorption of bovine serum albumin onto COP increased in the presence of kosmotropic salts, whereas adsorption of IgG increased in the presence of chaotropic salts. By contrast, the adsorption of these 2 proteins to PSt was unaffected by these Hofmeister salts. Langmuir-Freundlich model of COP adsorption suggested that the COP surface is more homogeneous for protein binding than the PSt surface. Furthermore, RIfS and sum frequency generation analyses indicated that water molecules bind more weakly to COP than to PSt. Our data propose a novel viewpoint of the way protein binds to COP surface that is different from the way it binds to PSt.

16. Phospholipid Membrane Fluidity Alters Ligand Binding Activity of a G Protein-Coupled Receptor by Shifting the Conformational Equilibrium

Yoshida K, Nagatoishi S, Kuroda D, Suzuki N, Murata T, and Tsumoto K.

The affinity of a ligand for a receptor on the cell surface will be influenced by the membrane composition. Herein, we evaluated the effects of differences in membrane fluidity, controlled by phospholipid composition, on the ligand binding activity of the G protein-coupled receptor human serotonin 2B. Using Nanodisc technology to control membrane properties, we performed biophysical analysis and employed molecular dynamics simulations to demonstrate that increased membrane fluidity shifted the equilibrium toward an active form of the receptor. Our quantitative study will enable development of more realistic in vitro drug discovery assays involving membrane-bound proteins such as G protein-coupled receptors.

17. Generation and characterization of antagonistic anti-human interleukin (IL)-18 monoclonal antibodies with high affinity: Two types of monoclonal antibodies against full-length IL-18 and the neoepitope of inflammatory caspase-cleaved active IL-18

Nariai Y, Kamino H, Obayashi E, Kato H, Sakashita G, Sugiura T, Migita K, Koga T, Kawakami A, Sakamoto K, Kadomatsu K, Nakakido M, Tsumoto K, and Urano T.

Interleukin-18 (IL-18) is a pro-inflammatory cytokine that evokes both innate and acquired immune responses. IL-18 is initially synthesized as an inactive precursor and the cleavage for processing into a mature, active molecule is mediated by pro-inflammatory caspases following the activation of inflammasomes. Two types of monoclonal antibodies were raised: anti-IL-1863-68 antibodies which recognize full-length1-193 and cleaved IL-18; and anti-IL-18 neoepitope antibodies which specifically recognize the new N-terminal 37YFGKLESK44 of IL-18 cleaved by pro-inflammatory caspase-1/4. These mAbs were suitable for Western blotting, capillary Western immunoassay (WES), immunofluorescence, immunoprecipitation, and function-blocking assays. WES analysis of these mAbs allowed visualization of the IL-18 bands and provided a molecular weight corresponding to the pro-inflammatory caspase-1/4 cleaved, active form IL-1837-193, and not to the inac-

tive precursor IL-18, in the serum of patients with adult-onset Still's disease (6/14, 42%) and hemophagocytic activation syndrome (2/6, 33%). These monoclonal antibodies will be very useful in IL-18 and inflammasome biology and for diagnostic and therapeutic strategies for inflammatory diseases.

18. Affinity Improvement of a Cancer-Targeted Antibody through Alanine-Induced Adjustment of Antigen-Antibody Interface

Yamashita T, Mizohata E, Nagatoishi S, Watanabe T, Nakakido M, Iwanari H, Mochizuki Y, Nakayama T, Kado Y, Yokota Y, Matsumura H, Kawamura T, Kodama T, Hamakubo T, Inoue T, Fujitani H, and Tsumoto K.

To investigate favorable single amino acid substitutions that improve antigen-antibody interactions, alanine (Ala) mutagenesis scanning of the interfacial residues of a cancer-targeted antibody, B5209B, was performed based on X-ray crystallography analysis. Two substitutions were shown to significantly enhance the binding affinity for the antigen, by up to 30-fold. One substitution improved the affinity by a gain of binding enthalpy, whereas the other substitution improved the affinity by a gain of binding entropy. Molecular dynamics simulations showed that the enthalpic improvement could be attributed to the stabilization of distant salt bridges located at the periphery of the antigen-antibody interface. The entropic improvement was due to the release of water molecules that were stably trapped in the antigen-antibody interface of the wild-type antibody. Importantly, these effects of the Ala substitutions were caused by subtle adjustments of the binding interface. These results will be helpful to design high-affinity antibodies with avoiding entropy-enthalpy compensation.

<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 higher-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 them in transmission electron microscope (TEM). But the technical difficulties discouraged us from such a troublesome procedure. 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 cut 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 terminals.

Aside from getting 3D information, it became possible to analyze much wider areas by using SEM to observe thin TEM sections than by using TEM. These methods are also applicable 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, 12 projects in 8 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[1]'s laboratory: [1]Divi-

sion of Molecular Virology, Department of Microbiology and Immunology, regarding the infection/replication processes of herpes simplex virus (HSV). Thin section electron microscopy has 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.

a-2. Roles of LPCAT1 in development and maintenance of photoreceptor outer-segment.

We have been performing several studies also with research groups in Dr. Watanabe[2]'s laboratory: [2]Project Division of Molecular and Developmental Biology. This year, we analyzed the role of LPCAT1 (Lysophosphatidylcholine acyltransferase 1) in retinal development. With the analysis of the retina by electron microscopy, we revealed that organized photoreceptor outer-segments are formed normally in *LPCAT1*-KO mouse retinas and then degrade, suggesting that LPCAT1 have roles in maintenance of photoreceptor outer-segments. Some other projects are also running with Dr. Watanabe's laboratory.

Some other collaborative research works using thin section electron microscopy and/or immuno-electron microscopy were performed with Dr. Noda[3], [3]Laboratory of Ultrastructural Virology, Department of Virus Research, Institute of Frontier Life and Medical Sciences, Kyoto University, Dr. Sasou[4], [4]Division of Mucosal Immunology, concerning function of intestinal Paneth cells in mucosal immunity, Dr. Eguchi[5], in [5]Division of Genetics, Dr. Nakahara[6] in [6]Department of Life Science Dentistry, The Nippon Dental University 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 were used to analyze exosomes in collaboration with Dr. Ishii[7] in [7]Division of Vaccine Science, Laboratory of Adjuvant Innovation. The same techniques are also used in the research with Dr. Shibata[8] in [8]Biomolecular Engineering Laboratory, Waseda University.

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. Chou[9], in [9]Department of Research and Development, Life Innovation Center, LIFEBANK Japan, Inc., about the morphology of multipotent stem cells.

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